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Human IgG and IgM monoclonal antibodies against autologous melanoma produced by Epstein-Barr-virus-transformed B lymphocytes*

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Summary. The serum antibody response to human melanoma has prognostic and potential physiological consequences. The specificity of the host B cell antibody response may be an important determinant of disease outcome. We have utilized Epstein-Barr virus (EBV) transformation to analyze the repertory of the host B cell response to melanoma. Production of antibody that binds selectively to autologous (eight cases) or allogeneic (four cases) short-term-cultured melanoma cells was assessed from EBV-transformed B lymphoblastoid cells. Forty-two cultures of EBV-transformed B cells that secreted IgM and 23 that secreted IgG antibodies gave patterns of differential reactivity with autologous or allogeneic melanoma. Antibody-forming B cells persisted in producing melanoma-reactive IgG and IgM for 8–21 weeks. Preselection of B cells by adsorption to tumor cell antigens before transformation enhanced the frequency of antibody secretion. The specificity of the antibody produced by the longest-producing culture appears to be restricted to a subset of melanomas. The patient from whom this tumor-restricted IgG-producing B cell was retrieved was unusual, having had a transient serum IgG of similar specificity, and having manifest a syndrome of vitiligo at the time of her development of serum antimelanoma antibody, followed by disease-free survival of resected recurrent metastatic melanoma to the present (more than 6 years). This study has given support to findings of conventional serology, revealing the production of melanoma-reactive antibody from B cells of patients who have demonstrable serological response to tumor.

Introduction

Substantial indirect clinical and pathological evidence, as well as direct serological studies [1, 16, 26, 27] and cellular investigations [5, 9, 10, 30], suggests that human melanoma cells may be immunogenic to the host of origin.

Transformation by the Epstein-Barr virus (EBV) provides an efficient means to immortalize human B cells and has been used to derive cell lines that secrete monoclonal antibodies to a number of microbial and cellular antigens [4, 17, 18, 20, 24, 28, 35]. EBV-transformed B cell lines that secrete IgM antibody to allogeneic melanoma have been isolated from the peripheral blood and tumor tissue [12, 13, 29, 33]; however, EBV transformation has not yet been used systematically to examine the repertory of the host immune response to autologous melanoma in any prospective series of patients. Antibody produced by two EBV-transformed B cell lines in one laboratory, and three in another, have revealed IgM antibodies without IgG [12, 33].

We have examined whether EBV transformation may reveal the nature of the host response to autologous melanoma at a clonal level and have focused our efforts upon a series of patients whose serological response to melanoma has previously been reported [16, 31]. The technique of EBV transformation provides an avenue for clonal insight into the antibody response of melanoma patients and the potential for the development of therapeutic or diagnostic antibodies in conjunction with other techniques to sustain the EBV-transformed B cell antibody response.

Materials and methods

Tumor and lymphocyte specimens. Lymphocytes were obtained from 8 patients with metastatic melanoma from whom tumor cell lines had been established and from 4 patients from whom tumor cell cultures were not available. The sources of tumor cell lines and B cell cultures in these subjects are listed in Tables 1 and 2. Monolayer autologous melanoma lines were studied in short-term cultures maintained for less than six passages in F-10 medium with 10% fetal calf serum (Gibco, Grand Island, NY) containing 1% penicillin and streptomycin [16]. Tumor cell cultures were characterized as melanoma by light and/or electron microscopy and by histochemistry for melanin and melanosomes or premelanosomes. All cultures were free of mycoplasma, fungal, and bacterial contaminants by culture and Hoechst stain number 33258 [2]. Of six

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Table 1. IgG- and IgM-secreting EBV-transformed B cell cultures obtained from 4 patients tested against allogeneic melanoma

Patient[a]	Source of B cells	No. wells plated	No. wells with initially melanoma-restricted antibody	
			IgG	IgM
1 (DA)	Regional lymph node	400	0	1
2 (NL)	Regional lymph node	200	0	0
3 (BN)	Blood	200	0	1
4 (BT)	Blood	400	2	1

[a] Locally recurrent Y-Mel 81 : 180 tumor employed for assay

melanoma cell lines, kindly karyotyped by A. Cunningham (Pfizer Corp., Groton, Conn.), all had a near-diploid complement of human chromosomes.

Lymphocytes were isolated by density centrifugation upon Ficoll/Hypaque from the peripheral blood of 10 patients and 3 controls. One donor was notable for having developed a transient serum antibody response to her autologous cultured melanoma cells during the onset of a syndrome of widespread vitiligo [31]. She had received intralymphatic treatment with the methanol extraction residue of tuberculin and 6 months of i. d. bacillus Calmette-Guérin following relapse 6 years ago and has remained well to date. Lymphocytes from 2 patients had been cryopreserved in liquid nitrogen. Freshly disaggregated lymph nodes were the source of lymphocytes from 2 other patients. T cells were depleted from the peripheral blood specimens of the first four cases and in the three normal donors using rosette formation with neuraminidase-treated sheep erythrocytes and sedimentation through Ficoll/Hypaque. In an experiment carried out with the melanoma line Y-Mel 82: 550 [16], lymphocytes binding to tumor cell antigens were preselected for transformation by one of two procedures. In one, lymphocytes were selected by adsorption to plastic dishes coated with a 1% concentration of sodium-dodecyl-sulfate(SDS)-solubilized autologous melanoma cell membranes; in the other, lymphocytes were incubated upon intact monolayers of viable autologous melanoma. Preselection was carried out by adsorption for 4 h at 37° C. Nonadherent cells were removed and the adherent population was detached mechanically, while an unselected population of the same lymphocytes was transformed and studied as a control. Another population of cells was cultured in purified B cell growth factor (Cellular Products, Buffalo, N.Y.) for 7 days prior to EBV transformation.

Transformation. Lymphocyte populations at a density of $(1-2)\times10^6$ cells/ml were inoculated with EBV from supernatant fluid from the B95-8 marmoset cell line for a period of 24 h at 37° C. EBV-inoculated cells were plated at a density of 500 cells/microwell in 96-well microtiter plates containing feeder layers of foreskin fibroblasts. Plates were incubated at 37° C in a humidified 5% CO_2 incubator with feeding at 7-day intervals. All cultures were maintained in RPMI-1640 medium plus 20% fetal calf serum and antibiotics.

Subcultures. Secretion of IgM or IgG antibody by the primary subcultures was first assessed at the 2nd or 3rd week following EBV transformation and thereafter at each feeding. Parallel enzyme-linked immunosorbent assay (ELISA) immunofiltration tests or micro-ELISA assays were carried out against melanoma cells and fibroblasts to determine the presence of differential reactivity to melanoma and then to select antibody-forming cells for subculture.

Expansion of B cell subcultures was carried out in 13×100-mm tubes. These cultures were fed at weekly intervals, and the supernatant fluids were harvested for immediate assay as well as frozen for later study. Transformed antibody-secreting B cells were cloned at 1–10 cells/microtiter plate well upon feeder layers of human foreskin fibroblasts, mouse peritoneal macrophages, or human cord blood lymphocytes.

Immunofiltration ELISA and micro ELISA. A 96-channel replica-plating device of our own design was used to transfer supernatant fluids from microwell cultures to corresponding wells of assay plates or empty plates for storage. The immunofiltration ELISA method of Glassy et al. [8] was modified to detect antibody to viable cultured cells. Target melanoma or other tumor cells, melanocytes, or fibroblasts were immobilized on glass filter discs placed in the wells of flat-bottomed 96-well microtiter plates perforated by drilling at the center of each well to allow washing upon a suction box fitted to the underside of the plate. The plates and filters were blocked in buffer containing 10% fetal calf serum, 1% bovine serum albumin (Sigma, St. Louis, Mo.), and 0.3% gelatin overnight at 4° C. Cells (2.5×10^4 of melanoma cells in one plate and fibroblasts in the other) were inoculated into each microwell and washed three times by suction in 0.3% gelatin/phosphate-buffered saline. The discs were not allowed to dry during the washing process. B cell supernatant fluids (0.05 ml) were added to each well for 1 h at room temperature. Plates were washed five times by suction. Isotype-specific peroxidase-conjugated goat antibodies to human immunoglobulins (Tago) were added to each well for 1 h at 37° C and washed five times. Finally, 100 µl 0.04% *o*-phenylenediamine, 0.01% H_2O_2 in 0.05 M citrate, and 0.1 M phosphate buffer (pH 5.0) were added to each well for 30 min at room temperature. The reaction was terminated by the addition of 0.05 ml 2 M H_2SO_4/well. A 200-µl sample of fluid from each well was transferred to an empty plate for absorbance reading at 492 nm in a Titertek Multiskan (Flow Laboratories, Irvine, Calif.). Background readings for B cell cultures producing an irrelevant immunoglobulin or no immunoglobulin yielded absorbance readings of $\leqslant 0.1$ in this assay. This assay has also been performed on a micro scale using adherent methanol-fixed target cells in 72-well 0.02-ml Teresaki typing plates with correspondingly smaller volumes of reagents and as few as 1000 target cells for screening.

We attempted to avoid overgrowth of irrelevant transformants and to improve the yield of stable melanoma-reactive IgG-forming EBV transformants by initiating cultures at low cell density, by early and repeated cloning of antibody formers, and by preselection of B cells on the basis of their ability to adsorb to melanoma antigens. Preselection successfully increased the proportion of antibody-producing cultures but none of these measures affected the duration of antibody secretion with melanoma specificity.

Results

In pilot studies, lymph node and peripheral blood B cell cultures from four patients were transformed in microwell cultures and tested for production of antibody against one allogeneic melanoma cell line (Y-Mel 81 : 180) previously demonstrated to exhibit a common melanoma cell-surface antigen [16]. As shown in Table 1, a single culture from 3 of the 4 patients produced IgM antibodies that bound to allogeneic melanoma but not fibroblasts, and two cultures from 1 patient secreted IgG antibodies. Diminished secretion of these antibodies occurred over time, and efforts to clone the antibody-forming transformants were unsuccessful.

The antibody response of peripheral blood B cells was examined here to determine whether B cells committed to synthesizing antibodies to cell-surface antigens of cultured melanoma are present and detectable in the circulation of patients with melanoma. IgG as well as IgM antibodies reacting with intact viable melanoma cells immobilized on glass-fiber filter disks were obtained from B cells of the peripheral blood or regional lymph nodes from 10 of 12 patients, including 7 of 8 patients who were studied in autologous test systems. The majority of these patients' B lymphoblasts produced IgM, but 2 also showed IgG production. The E_6D_3 monoclonal cell line derived from one patient exhibited relatively prolonged IgG production for

Table 2. IgG- and IgM-secreting EBV-transformed B cell cultures obtained from 8 patients, tested against autologous cultured melanoma cells

Patient	Tumor	Tumor sites	Peripheral blood B cell source	No. of wells plated	No. wells with initially melanoma-restricted antibody	
					IgG	IgM
5 (S)	Y-Mel 80:130	Regional lymph node	Cryopreserved	200	0	1
6 (Y)	Y-Mel 80:180	Bowel metastasis	Cryopreserved	200	0	1
7 (L)	Y-Mel 81:692	Bowel metastasis	Fresh	200	0	0
8 (N)	Y-Mel 81:280	Primary tumor	Fresh	200	0	1
9 (H)	Y-Mel 82:820	Soft tissue metastasis	Fresh	200	0	1
10 (G)	Y-Mel 81:710	Brain metastasis	Fresh	400	0	6
11 (B)	Y-Mel 83:070	Intransit metastasis	Fresh	200	2	4
12 (Z)	Y-Mel 82:550	Local recurrence	Fresh	400	9	7
			Fresh[b]	1400[a]	12	21

[a] Feeder layer of mouse peritoneal macrophages

[b] Summary results with and without preselection upon melanoma cell monolayer or lysate (see Table 4)

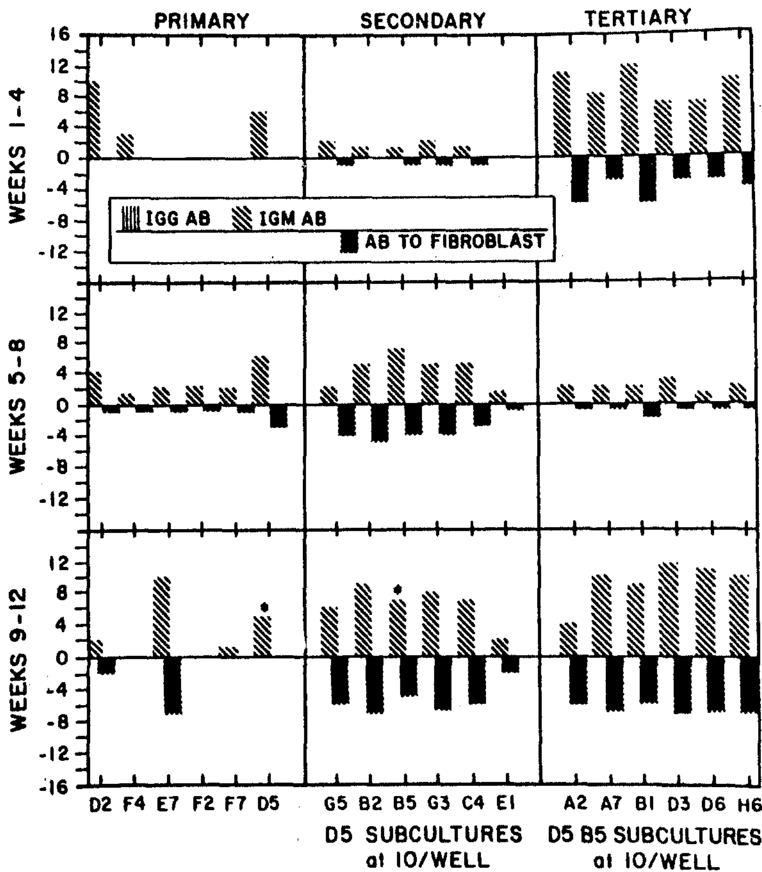

Fig. 1. Serial studies of IgM antibody produced by clones of Epstein-Barr-virus(EBV)-transformed B cells from patient 12 against the autologous melanoma Y-Mel 81:710. Transformants were selected for antibody production to melanoma, and non-reactivity to fibroblasts in the 1st–4th weeks of culture. Melanoma reactivity of the individual B cell cultures is indicted by the histograms displayed above the baseline (+), and fibroblast reactivity by histograms displayed below the baseline (–); * Indicates culture studied in further subculture

at least 20 weeks, providing supernatant antibody sufficient for direct specificity analysis by several methods including ELISA, protein A hemadsorption, anti-IgG hemadsorption, and a preliminary absorption analysis. Specificity of this IgG-forming B cell culture appears to be directed against antigens detected by this patient's native serum [31] previously reported. Reactivity was demonstrated against two of seven short-term-cultured melanoma cell lines, but not against normal fibroblasts, melanocytes, or carcinomas of breast or pancreas. Thus, the E6D3 B cell product detected in vitro was similar in specificity to serum antibodies previously reported to be transiently detectable in the serum of this patient [31], detecting a tumor-restricted cell-surface antigen of melanoma. Control studies with the B cells of three normal donors (who lacked serum antibody reactivity against melanoma) failed to reveal sustained production of any antibody that demonstrates differential melanoma reactivity. Limited amounts of B cell culture fluids containing antibody from other melanoma patients' antibody-producing lines could only be tested directly in the initial serial screening tests.

A total of 3400 EBV-inoculated lymphocyte cultures were established from 8 patients whose autologous melanoma cell cultures were available in sufficient quantity to be used as antigen in the immunofiltration ELISA. Results obtained from screening these cultures for antibody pro-

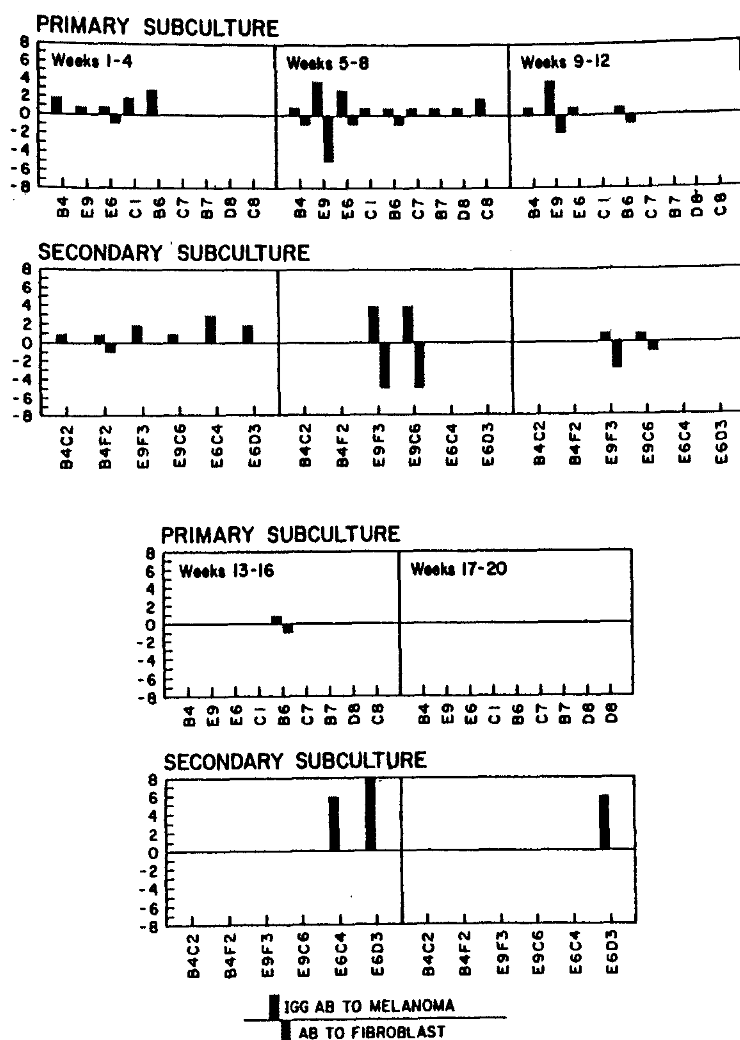

Fig. 2. Serial studies of IgG antibody produced by a series of cloned EBV-transformed B cell cultures of patient 12 against the autologous melanoma Y-Mel 82:550. The antibody reactivity of primary and secondary subcultures tested in five periods of 4 weeks each are displayed, (as in Fig. 1) with persistence of melanoma reactivity in one clone at weeks 17–20 (E_6D_3). An analysis of the specificity of the antibody produced by E_6D_3 is presented in Table 3

duction to autologous melanoma are presented in Table 2. One patient yielded no antibody-producing B cell cultures. A single B cell line that produced IgM, which bound to autologous tumor but not fibroblasts, was obtained from each of 4 patients (pts. 5, 6, 8, 10). These lines were propagated in culture tubes and, without being cloned, continued to produce melanoma-reactive fibroblast-nonreactive IgM antibody over a period of 5–16 weeks. Antibody from the D_7 cell line of patient 6 (Y) reacted only with melanoma throughout 12 weeks of culture; however, subsequent culture fluids collected between 13 and 16 weeks contained reactivity against both melanoma and fibroblasts.

During the initial screening of transformed B cell cultures from patient 10, three cell lines (D_2, F_4, and D_5) produced melanoma-reactive fibroblast-nonreactive IgM antibodies. During the 5th–8th weeks these cultures, along with three additional cultures (E_7, F_2, and F_7), produced antibody that reacted with melanoma, but weak reactivity with fibroblasts, which was clearly above background, was also observed. Between 9 and 12 weeks, four of these cell lines continued to secrete antibody; antibody from two lines was both melanoma- and fibroblast-reactive, while antibody from two others was only melanoma-reactive. One of these lines (D_5) was successfully cloned twice at limiting dilution but all clones subsequently produced IgM antibody that bound to both melanoma and fibroblasts indiscriminately. As shown in Fig. 1, five IgM-producing clones emerged, none with differential binding to melanoma as compared to fibroblasts. Studies in patient 11 yielded transient production of melanoma-reactive fibroblast-nonreactive IgM in four cultures.

Cultures from patients 10, 11, and 12 produced IgG as well as IgM antibody that bound preferentially to autologous melanoma (Table 2). A total of 16 cell lines were established from patient 12, producing antibodies that bound to autologous melanoma. Patient 12 was unusual in that 9 of her 16 cultures were found to produce IgG antibodies. In 5 of these, the antibody was reactive only against melanoma, while in 4 it was both melanoma-reactive and, to a lesser degree, fibroblast-reactive at some time in the first 12 weeks (Fig. 1). We attempted to segregate populations of transformants that secreted antibodies of more restricted specificity by cloning three cell lines B_4, E_9, and E_6 (Fig. 2). Clones of E_9 produced antibody equally reactive to fibroblasts and melanoma while one clone of B_4, (B_4C_2), gave antibody of more restricted reactivity to melanoma than the other (B_4F_2).

Two clones of the E_6 cell line were derived that secreted melanoma-reactive IgG of interest. E_6D_3 pro-

Table 3. Specificity analysis of E_6D_3 antibody by protein A and anti-IgG hemadsorption and enzyme-linked immunosorbent assay

Culture	Tests on EBV-transformed B cell clones[a]		
	PA-HA	aIgG-HA	ELISA[c]
Y-Mel			
82:550	+	+	+
81:710	±	–	–
81:180	–	–	–
80:130	–	–	–
84:240	–	–	–
84:280	–	–	
84:340	–	–	
Non-malignant cell cultures			
Fibroblasts[b]	–		–
Melanocytes[b]	–	–	–
Malignant non-melanoma cultures			
Breast[b]	–	–	–
Pancreas[b]	–	–	–

[a] PA-HA and aIgG-HA, protein A and anti-IgG hemadsorption test reactivity: scored as ++ if >5% positive cells and scored + if >2% but <5% positive cells

[b] Cultured cells cell lines used for these tests were fibroblasts (PF-1) carcinoma of breast (CAL-18), pancreas (COLO-8), melanocytes (FSC-8)

[c] ELISA tests were scored as + if A_{492} readings were >0.10; negative if readings were <0.10; background readings consistently <0.030

duced an IgG antibody binding to melanoma but not to fibroblasts in relatively large amounts until the 8th week. Up to 10 μg/ml antibody production in E_6D_3 and E_6C_4 could be detected in the supernatants of these cultures for a period of 9 weeks, after which melanoma-reactive IgG antibody was again detectable in both E_6D_3 and E_6C_4 in the 13th week. Supernatant fluids from the E_6D_3 clone collected for the next 12 weeks contained antibody that was further characterized. The analysis of light-chain type in this culture revealed reactivity only with anti-κ reagents at the peak of antibody production (16 μg/ml) between weeks 12 and 20.

The specificity of E_6D_3 antibody was analyzed by immunofiltration ELISA, protein A hemadsorption, and anti-IgG hemadsorption. The antibody reacted with the autologous melanoma Y-Mel 82:550 and one of six other short-passaged melanoma lines (Y-Mel 81:710). It was nonreactive with plascental fibroblasts, foreskin melanocytes [4], breast carcinoma (CAL-18), and pancreatic carcinoma (COLO-8) cell lines. These data are summarized in Table 3.

The absorption analysis of E_6D_3 was limited due to the amounts of antibody available. Absorption at 17°C for 40 min [16] with 1:1 v:v packed cells of the autologous 82:550 melanoma removed all reactivity of E_6D_3 while similar absorption with melanoma line 81:180, fibroblasts, and the autologous bulk EBV-transformed B cells failed to diminish reactivity of E_6D_3 to the autologous 82:550 melanoma cell culture.

Preselection of B cells

We attempted to enhance the frequency, durability, and specificity of antibody formation in this system by preselecting for B cells bearing Ig receptors for autologous melanoma upon plastic petri plates coated with a lysate prepared using SDS from autologous Y-Mel 82:550; alternatively, B cells were selected by adsorption to monolayers of this melanoma. Feeder layers for transformed B cells in this experiment were mouse peritoneal macrophages rather than human foreskin fibroblasts, as in the previous experiments. The results of this experiment are shown in Table 4. The frequency of transformants producing IgM and IgG with differential binding to autologous melanoma was enhanced tenfold by preselection upon solubilized melanoma cell antigen (group A) compared to unselected (group D) or nonadherent (negatively selected) populations of lymphocytes (obtained in groups E or F). The positively selected B cell populations (A + B) gave four IgG and nine IgM antibody – formers in primary subculture, a frequency of 89–450/10^6 lymphocytes in the positively selected populations. This compares with 0–20 in the negatively selected population (E + F). The duration of antibody secretion by transformants arising from positively selected populations of lymphocytes was not, however, greater than had been observed in earlier experiments.

B cell antibody studies from normal donors

The possibility that B cells of normal donors might produce antibody with reaction specificities that are similar to

Table 4. Incidences of antibody-producing B cells in populations preselected by adherence to melanoma cell monolayers or lysates prior to transformation

Cell population transformed	Group	No. cells plated[a]	No. antibody (+)		Incidence of antibody-transformed (+) cells/1×10^6 lymphocytes	
			IgG	IgM	IgG	IgM
Autologous melanoma membrane						
Adherent	(A)	1.14×10^4	3	4	260	350
Nonadherent	(E)	2.00×10^5	3	4	15	20
Autologous melanoma cell monolayer						
Adherent	(B)	1.12×10^4	1	5	89	450
Nonadherent	(F)	2.0×10^5	0	2	0	10
Control unselected[b]	(D)	2.0×10^5	5	6	25	30

[a] An identical number of wells was plated in each group, with lymphocyte numbers varying according to cells retrieved

[b] Groups D and C were equivalent

those detected among melanoma patients was tested by EBV transformation of peripheral blood B cells from five normal volunteers aged 25–35 years. Parallel ELISA tests were carried out upon fibroblasts and at least two melanoma cell lines (one of which was the Y-Mel 82:550 line, which had been the autologous melanoma reactive with E_6D_3, and the other of which was Y-Mel 84:420). No sustained melanoma-reactive fibroblast-nonreactive antibody was detected by ELISA among 1320 cultures (2×10^4 transformed peripheral blood B cells each) from these five donors. One IgM-producing culture was detected that showed a differential melanoma-reactive pattern for 5 weeks, with loss of this reactivity thereafter and a nonspecific reactivity to melanoma and fibroblasts following the 9th week.

Discussion

Antibody to cultured autologous melanoma cells can be infrequently detected in the serum of melanoma patients and has generally been of low titer [1, 16, 26, 27]. Cell-surface antigens of melanoma have also been defined by the use of hetero-antisera or mouse monoclonal antibodies; however, antigens identified by these murine reagents have not been shown to play any role in the host-tumor interaction. Human-murine and human-human hybridoma antibodies offer potential future alternatives for the dissection of the autologous human immune response to cancer for the future that are as yet largely unrealized [3, 15, 34].

The relevance of in vitro antibody secretion to the natural history of melanoma is suggested by the fact that both of the peripheral blood samples that gave B cell lines secreting IgG antibodies to autologous melanoma were derived from patients who had serum IgG antibody reactivity to their tumors [16, 31]. It is of note that one of these patients (the donor of Y-Mel 82:550 and the E_6D_3 lymphoblastoid B cell line) developed a systemic syndrome of widespread vitiligo coincidental with her development of a transient serum IgG antibody, and has remained free of disease to date, 6 years after surgical removal of recurrent metastatic disease [31]. The survival of this patient with a high-risk melanoma suggests a favorable prognostic significance of her antibody response. This is consistent with the findings of Jones et al. [14], but at variance with results obtained in the setting of a prospective controlled adjuvant trial, where serological reactivity was assessed using a common melanoma-antigen-bearing cell line [32]. At the time of peripheral blood sampling for EBV transformation in this study, serum antibody was no longer detectable in this patient. Taken together, these considerations suggest that EBV transformation affords investigators a means to retrieve physiologically relevant B cell responses to melanoma and other cancers and to dissect these at a clonal level, even when serum antibody no longer allows one to detect the host response.

Production of melanoma-reactive IgG antibodies by autologous or allogeneic EBV-transformed B cells has not been previously reported. The larger numbers of IgM-forming EBV transformants observed in our study, and their exclusive detection in several earlier studies of melanoma patients using EBV, may be explained by the preferential outgrowth or numerical predominance of IgM- over IgG-committed B cells, or by a greater susceptibility of IgM secretors to EBV transformation [12, 13, 29, 33]. The report of Watson et al. [33] concerning in vitro growth of B lymphocytes infiltrating melanoma metastases is significant because one of the transformed lymphoid cell lines tested produced an IgM antibody reactive with autologous tumor. Studies by Irie et al. [12, 13] and Tai et al. [29] demonstrated IgM antibody production against a common oncofetal antigen in B cells from individuals with documented positive serological assays against the melanoma cell line M14. Since M14 expresses both GM_2 and GD_2, the presence of serum IgM reactive with M14 correlated with the isolation of L72 and L55, which detect the glycolipids GD_2 and GM_2 respectively (GM_2 being widely distributed in melanomas, breast cancer, sarcomas, and brain tumors) [12, 13, 29].

The major problem encountered in these pilot studies was the loss of detectable antibody production by EBV-transformed B cell lines. This problem is not unique in the present studies, having been noted by many other laboratories attempting to use EBV to produce antibodies against antigens in other systems [4, 20, 23, 24]. In yet other studies [4, 17, 18, 20, 24, 28, 35], as well as our own [7, 22], stable IgM and IgG production against a wide variety of antigens has been reported. Potential explanations for the loss of antibody production include: (a) growth instability due to lack of growth factors or nutritional requirements, or appropriate immunoregulatory or antigenic stimuli, or the occurrence of a lytic viral replicative cycle; (b) secretory instability related to the foregoing considerations, mutations in V-region genes, which alter the antibody-combining site, or imbalances in heavy- and light-chain synthesis as documented by Kozbor et al. [19]; or (c) overgrowth by irrelevant transformants.

We attempted to avoid overgrowth in this study by utilizing low-density cell cultures, by cloning antibody formers repeatedly, and by preselecting melanoma-antigen-adsorbent B cells. Preselection increased the proportion of antibody-producing cultures, but the duration of antibody secretion was not affected by any of these measures. Therefore, the problem of unstable antibody production does not appear to be one of overgrowth by irrelevant cells, but rather a problem intrinsic to the individual B cell clones of the isotypes we have isolated, and may correlate with the antigenic specificities against which they are directed. Whether antibody production by EBV-transformed cells can be stabilized or enhanced by somatic cell fusion with suitably selectable human or mouse cell lines remains to be established [6, 15, 19, 21, 25, 34].

As a consequence of the increased evidence for immunogenicity with primary melanoma, we have begun to focus efforts upon the host response to melanocytes from these earlier phases of melanocytic neoplasia, transforming B cells found in association with them. The use of hyperimmunization as a means to augment the yield of antibody producers is raised by the results of Kan-Mitchell et al. [15], and Yamaguchi et al. [34], who have employed EBV transformation followed by secondary fusion to obtain melanoma-reactive antibodies. In the latter study, antibody

IgG of identical specificity was reported to occur in serum and to be produced by B cells harvested from one patient who had previously been vaccinated against autologous tumor. The biological relevance of the antibodies produced in vitro by melanoma patients' B cells, and of antibodies detected in serum, remains to be established. Normal donor's sera have occasionally been shown to yield high titers of serological reactivity to melanoma [11], introducing the question of whether this is related to the presence of occult melanocytic disorders. Systematic studies of B cell antibody response in conjunction with serology and clinical evaluation for melanoma and dysplastic nevi may shed light upon these questions.

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