A Strategy for the Production of Human Monoclonal Antibodies Reactive with Lung Tumor Cell Lines

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ABSTRACT

Epstein-Barr virus (EBV)-immortalized cell lines were established from lymphocytes derived from peripheral blood, draining lymph nodes, bone marrow aspirates, tumors, and pericardial effusions from lung cancer patients. Ten of these lines were cloned and screened against glutaraldehyde-fixed lung tumor cells for tumor-specific antibody production using an enzyme-linked immunosorbent assay. None of the 140 clones tested secreted specific antibody, suggesting that B-lymphocytes specific for tumor antigens are rare in lung cancer patients. The EBV lines from the lung cancer patients were then hybridized with a thioguanine-resistant, ouabain-resistant human B-lymphoblastoid fusion partner KR-4, in an attempt to rescue low frequency B-cell precursors. Supernatants from more than 4500 hybridomas surviving hypoxanthine–aminopterin–thymidine:ouabain selection were screened against human lung tumor cells in an enzyme-linked immunosorbent assay. Over 360 hybrids showed significant levels of activity, although most were not tumor cell specific since they also reacted with EBV-infected cells from the lymphocyte donor. Two hybridomas showed apparent specific binding early after fusion, but this activity was lost upon continued growth although, in general, hybrids continued to secrete high levels of immunoglobulin M (up to 50 µg/ml), in some cases, beyond 1 year in culture. The human EBV-hybridoma system described here may be useful for rescuing low-frequency tumor-reactive B-cell precursors in lung cancer patients.

INTRODUCTION

Dramatic reductions in tumor load have been reported in cancer patients treated with murine MABs (15–17, 20, 21). However, the major impediment to long-term therapy has been the induction of an anti-mouse immunoglobulin response in these patients which neutralizes the effect of the monoclonal, anti-tumor antibodies (13). It is predicted that human MABs would be far less immunogenic, and therefore, much effort has been directed toward the development of a human hybridoma system (4, 12, 19). We have developed a system whereby donor lymphocytes are transfected with Epstein-Barr virus (EBV) and then hybridized with our 6-thioguanine-resistant, ouabain-resistant fusion partner, KR-4 (11). The fusion frequencies, stability, immunoglobulin production levels, convenience, and reliability of this system are greater than reported in other systems not using EBV (3, 12). We have therefore taken this approach to establish human hybridomas against human lung cancer cells.

A number of laboratories have reported the production of murine MABs with apparent specificity to human lung cancer antigens. These hybridomas were constructed from splenocytes of mice that had been immunized with lung tumor cell lines (1, 2, 6, 10), or fresh lung tumor in the form of intact cells or membrane preparations (1, 14). Methodologies used in making human MABs to lung cancer antigens have included interspecies hybrids of lymphocytes from lung cancer patients fused with rat or mouse myelomas (23) and hybrids of human lymphocytes fused with a human lymphoblastoid cell line (22). In addition, an attempt has been made to isolate specific human antibodies by EBV transformation of lymphocytes from the lymph nodes draining human lung cancers (9).

Due to the rareness of antitumor B-cell clones and severe limitations in the techniques used by these investigators, no strictly tumor-specific hybridomas could be generated. We now report on a new system which has the potential to generate tumor-specific human hybridomas in lung cancer and possibly other human cancers.

MATERIALS AND METHODS

Cell Lines. NCI-H69 (small cell lung cancer) and NCI-H209 (small cell lung cancer) were generously provided by J. Minna of the National Cancer Institute, Bethesda, MD. Tumor cell lines SK-LC-6 (large cell anaplastic lung cancer), SHP-77 (large cell variant of small cell lung carcinoma), SKMES-1 (squamous lung cancer), SK-LU-1 (adenocarcinoma of the lung), and AS49 (adenocarcinoma of the lung) were obtained from the Human Tumor Cell Laboratory of the Sloan-Kettering Institute for Cancer Research, Rye, NY. Tumor cell line DB (large cell anaplastic) was established from a biopsy sample from a 70-year-old man and characterized as described elsewhere.6 B lymphoblastoid cell lines were established by EBV infection of peripheral blood, regional lymph node, and tumor-infiltrating lymphocytes obtained from lung cancer patients. Peripheral blood lymphocytes were isolated by centrifugation on Ficoll-Hypaque (density, 1.077 g/ml; Pharmacia Fine Chemicals, Inc.); lymph nodes and tumor specimens were finely minced under sterile conditions and either passed through a fine-gauge wire sieve or, less frequently, treated with 0.5% Dispase (Boehringer Mannheim) in PBS to obtain single-cell suspensions; dead cells were removed by Ficoll-Hypaque sedimentation. EBV was obtained from Dr. Lau, Showa University Research Institute, Clearwater, FL, and added to lymphocytes at approximately 102 transforming units/cell. Stocks of established EBV lines were maintained at ~70° in RPMI 1640 supplemented with 20% FBS and 10% dimethyl sulfoxide. KR-4 is a ouabain-resistant (OuaR) mutant of the 6-thioguanine-resistant (ThgR) human lymphoblastoid cell line, GM 1500 (5), established from a biopsy sample from a 70-year-old man and characterized as described elsewhere.6 B lymphoblastoid cell lines were established by EBV infection of peripheral blood, regional lymph node, and tumor-infiltrating lymphocytes obtained from lung cancer patients. Peripheral blood lymphocytes were isolated by centrifugation on Ficoll-Hypaque (density, 1.077 g/ml; Pharmacia Fine Chemicals, Inc.); lymph nodes and tumor specimens were finely minced under sterile conditions and either passed through a fine-gauge wire sieve or, less frequently, treated with 0.5% Dispase (Boehringer Mannheim) in PBS to obtain single-cell suspensions; dead cells were removed by Ficoll-Hypaque sedimentation. EBV was obtained from Dr. Lau, Showa University Research Institute, Clearwater, FL, and added to lymphocytes at approximately 102 transforming units/106 cells. Stocks of established EBV lines were maintained at ~70° in RPMI 1640 supplemented with 20% FBS and 10% dimethyl sulfoxide. KR-4 is a ouabain-resistant (OuaR) mutant of the 6-thioguanine-resistant (ThgR) human lymphoblastoid cell line, GM 1500 (5), established in our laboratory (11) and is available for distribution. All cell lines were

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4 Recipient of grants from the Natural Sciences and Engineering Research Council, the Terry Fox Special Initiatives Program of the National Cancer Institute of Canada, and the Medical Research Council of Canada.
5 The abbreviations used are: MAb, monoclonal antibody; PBS, phosphate-buffered saline (137 mM NaCl-1.47 mM KH2PO4-8 mM Na2HPO4); HAT, hypoxanthine (100 µM)—aminopterin (0.4 µM)—thymidine (16 µM); FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; EBV, Epstein-Barr virus.

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routinely maintained in RPMI 1640 medium (Grand Island Biological Co.) supplemented with 10% FBS, 2 mM L-glutamine, and 50 µM 2-mercaptoethanol (Sigma Chemical Co.).

Cell Fusion and Hybrid Selection. Five to 7 days prior to fusion, KR-4 was cultured in tissue culture medium supplemented with ouabain (0.5 mM; Sigma) and 6-thioguanine (30 µg/ml; Sigma) to counterselect possible non-hybrid cells were removed 24 to 48 hr before fusion. EBV cell lines were maintained at a density of 1.0 x 10⁴ cells/ml or less. KR-4 (10⁴) and EBV line cells (10⁴) were washed in serum-free medium and the cells fused at room temperature using 0.5 ml of 45% (w/v) polyethylene glycol (Mw, 4,000; Sigma) in RPMI 1640 medium (pH 7.4) as described previously (11). Fused cells were gently washed and suspended in hydridoma medium [RPMI 1640 supplemented with 20% FBS, 2 mM L-glutamine, 50 µM 2-mercaptoethanol (Sigma), and gentamicin (50 µg/ml)]. The cells were plated into 96-well flat-bottomed microtiter plates (Linbro 76-032-05) in a total volume of 0.1 ml/well at 1 to 2 x 10⁴ cells/well with 3000-R-irradiated mouse spleen cells (2 to 5 x 10⁵ cells/well) and unirradiated mouse peritoneal exudate cells (5 x 10⁵ cells/well). Twenty-four hr after fusion, 0.1 ml of 2x HAT hybridoma medium (Flow Laboratories) containing 10 µg ouabain was added to each well without removing any media. Three days postfusion, approximately one-half the wells were washed and replaced with 0.1 ml of fresh 1x HAT hybridoma medium containing 10 µg ouabain. Thereafter, the cells were fed as required every 4 to 6 days in HAT:ouabain medium. Control cultures of parental cell lines (KR-4 and EBV cell lines) were included with each fusion, and no living cells could be detected after 10 to 14 days in HAT:ouabain medium. Putative hybrid cells were screened for reactivity with tumor cell lines 16 to 22 days after fusion.

Screening of Hybrids. Supernatants were screened using an ELISA against gluteraldehyde-fixed allogeneic, and, if available, autologous lung tumor cell lines. Supernatants were initially screened for reactivity against the human tumor cell line most appropriate to the source of donor lymphocytes; e.g., hybrids obtained using EBV-transformed lymphocytes from a patient with large cell lung carcinoma were initially and simultaneously screened against large cell tumor lines SK-LC-6, DB, and SHP-77 as well as the autologous EBV cell line. Nonadherent target cells (small cell lung cancer lines and EBV cell lines) were prepared for plating and fixation by washing twice with PBS, while adherent cells were first harvested with 0.02% EDTA in PBS and then washed. Target cells (10⁴/well) were fixed to polyvinyl chloride 96-well microtiter plates (Falcon 3092) using 0.25% glutaraldehyde (Sigma) in PBS as described by Minna et al. (18). Prior to layering on hybridoma supernatants, the plates were washed 3 times with PBS and 100 µl of 1% bovine serum albumin (radioimmunoassay grade; Sigma) in PBS added to each well. Supernatant (25 µl) was added to the wells and incubated for 1 hr at room temperature. Negative controls included RPMI:20% FBS, KR-4 supernatant, and human IgM (10 µg/ml; Cappel Laboratories). The plates were washed 3 times with PBS and then goat anti-human Ig (G + M + A) conjugated to horseradish peroxidase (50 µl) (affinity purified; Zymed) diluted 1:1000 in 1% bovine serum albumin in PBS was added to the wells. Following incubation at room temperature for 1.5 hr, the plates were washed 4 times with PBS and a substrate solution (100 µl) was added. The substrate solution was made immediately before use from a stock reagent of o-phenylenediamine (BDH Biochemicals Ltd.) (10 mg/ml in methanol), 50 mM citrate buffer (pH 5.0), and 3% H₂O₂ (1:99:0.1). The plates were incubated in the dark for 30 min, and the reaction was stopped with 8 x H₂SO₄ (25 µl). The absorbance differences between 492 and 405 nm were determined on an ELISA plate reader (Flow Laboratories). For quantitation of human immunoglobulin, polystyrene microtiter plates were coated with F(ab')2 fragment of goat anti-human immunoglobulin (10 µg/ml; Cappel) and non-specific binding blocked by a 1-hr incubation with RPMI 1640:10% FBS. Hybridoma supernatants were added, and antibodies that bound were detected with heavy-chain-specific goat anti-human IgG or anti-lgM conjugated with alkaline phosphatase (Zymed). Amounts of immunoglobulin were calculated by interpolation of a standard curve (range, 1 ng/ml to 10 µg/ml) constructed with affinity-purified human IgG and IgM (Cappel).

RESULTS AND DISCUSSION

EBV-immortalized cell lines were successfully established from lymphocytes derived from a variety of patients exhibiting all of the major histological types of lung cancer (Table 1). The time required to grow sufficient numbers of EBV-transformed cells for fusion varied from patient to patient and ranged from about 10 days to 6 weeks post-EBV infection. EBV transformation of tumor-infiltrating lymphocytes usually took much longer, but this may be attributed to the fact that there were probably fewer lymphocytes in the original total cell population infected.

Several attempts were made to clone the EBV lines from 10 patients by limiting dilution. The lines originated from peripheral blood (Patients MC, BR, BZ, KD, CM, and PV), tumor-draining lymph nodes (BG, GR, and BE) and a pericardial effusion (GL). In these experiments, less than 4% of cells came up positive for growth when seeded at 1 cell/well, and the presence of irradiated mouse spleen feeder cells did not improve the results. In addition, there was considerable variability in the cloning efficiency of the different lines since some lines (KD-P-EBV and CM-P-EBV), exhibited no growth at 1 cell/well even in repeated experiments, whereas MC-P-EBV consistently gave rise to about 3% growth. Not surprisingly, EBV lines cloned at higher cell densities showed an increase in cloning efficiency (Table 2; Chart 1) which again varied between lines. We then asked if the EBV line clones could secrete anti-tumor cell antibodies.

Supernatants from all the clones obtained were tested in an ELISA for tumor specificity by screening for a positive reaction on gluteraldehyde-fixed tumor cells and for a negative reaction on the matched EBV cells treated in a similar fashion. Of 140 clones tested, only one clone from cell line BZ-P-EBV showed reactivity with NCI-H69 tumor cells. However, this clone also reacted with BZ-P-EBV cell lines and therefore was not tumor cell

<table>
<thead>
<tr>
<th>Tumor histology</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small cell</td>
<td>KD, BR, BZ, MC, CM, HI, PW, LB, NC, OM, LV, WL (peripheral blood)*</td>
</tr>
<tr>
<td></td>
<td>AD, WL, JO-2 (bone marrow)</td>
</tr>
<tr>
<td>Large cell</td>
<td>GL (pericardial effusion)</td>
</tr>
<tr>
<td></td>
<td>SL, MK (lymph node)</td>
</tr>
<tr>
<td></td>
<td>KD (tumor)</td>
</tr>
<tr>
<td></td>
<td>DB, KD (peripheral blood)</td>
</tr>
<tr>
<td>Squamous</td>
<td>MN (lymph node)</td>
</tr>
<tr>
<td></td>
<td>WB, BT, JO, KX (tumor)</td>
</tr>
<tr>
<td>Bronchoalveolar</td>
<td>BG (lymph node)</td>
</tr>
</tbody>
</table>

* Tumors were classified by histological and morphological criteria as outlined by WHO.

Table 2

Cloning of EBV lines

<table>
<thead>
<tr>
<th>Cell density¹ (cells/well)</th>
<th>Wells positive for growth*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0/672 (0)²</td>
</tr>
<tr>
<td>10</td>
<td>10/384 (3)</td>
</tr>
<tr>
<td>60</td>
<td>83/384 (22)</td>
</tr>
</tbody>
</table>

* Wells were scored for cell growth 4 weeks after seeding.

¹ Numbers in parentheses, percentage.
Supernatants from over 4500 hybrids were screened against lung tumor cells, and over 360 showed some level of activity. Most of these antibodies were not tumor cell specific since they also reacted with EBV-infected cells from the lymphocyte donor. Supernatants from 2 hybridomas (BZ-11 and BZ-13), when tested 17 days postfusion, showed reactivity with tumor cells and did not react with matched EBV cells. However, this apparent specificity was lost by 27 days postfusion (Table 5). Although BZ-11 lost its specificity, it still maintained IgM production of greater than 50 μg/ml when measured 65 days postfusion. Loss of specific antibody production is probably due to the overgrowth of nonspecific or nonproducing hybridomas and could be avoided by early cloning of hybrids. Hybrid BR-1, BR-2, and BR-3 (Table 5), although not tumor specific, produced levels of IgM ranging from 0 to 7% (Table 4). and did not react with matched EBV cells. However, this apparent specificity was lost by 27 days postfusion (Table 5). Although BZ-11 lost its specificity, it still maintained IgM production of greater than 50 μg/ml when measured 65 days postfusion. Loss of specific antibody production is probably due to the overgrowth of nonspecific or nonproducing hybridomas and could be avoided by early cloning of hybrids. Hybrid BR-1, BR-2, and BR-3 (Table 5), although not tumor specific, produced levels of IgM ranging from 0 to 7% (Table 4).

The results from 103 fusions are presented in Table 3. The percentage of wells showing growth after HAT:ouabain selection ranged from 57 to 97% depending on the EBV line fused. The percentage of hybrids secreting antibody reactive with lung tumor cells ranged from 0 to 20%. This latter finding appears to be influenced by at least 2 factors: (a) the source of lymphocytes which have been immortalized with EBV; EBV lines derived from tumor-draining lymph nodes appear to yield a greater percentage (13 to 20%) of hybrids secreting antibody reactive with tumor cells than do EBV lines derived from peripheral blood (0 to 7%) (Table 4); and (b) it was noted that with increasing passage number, EBV lines yielded fewer hybrids secreting antibody reactive with tumor cells, although the fusion frequency itself was unaffected (data not shown).

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One of the main limitations of EBV-transformed lymphocytes as a source of human MAb is the relatively low quantities of antibody produced and the fact that antibody production declines with cell passage (3, 12). To circumvent these limitations, we developed a technique for hybridizing EBV-transformed lymphocytes with a human fusion partner to yield hybridomas (11). This hybridization serves to stabilize and increase antibody production of the EBV-transformed cells. In the present study, the EBV lines of lung cancer patients were fed with our ThgR, OuaR, human B-myeloma IgM (100 μg/ml) were less than 0.025.

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Table 5

<table>
<thead>
<tr>
<th>Hybrida</th>
<th>Cell lines testedb</th>
<th>17 days</th>
<th>27 days</th>
<th>33 days</th>
<th>63 days</th>
<th>IgM (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR-1</td>
<td>NCI-H69</td>
<td>0.277</td>
<td>0.485</td>
<td>1.073</td>
<td>0.619</td>
<td>50</td>
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<tr>
<td></td>
<td>BR-P-EBV</td>
<td>0.261</td>
<td>0.298</td>
<td>0.836</td>
<td>0.648</td>
<td></td>
</tr>
<tr>
<td>BR-2</td>
<td>NCI-H69</td>
<td>0.125</td>
<td>0.268</td>
<td>0.211</td>
<td>0.012</td>
<td>1.99</td>
</tr>
<tr>
<td></td>
<td>BR-P-EBV</td>
<td>0.101</td>
<td>0.127</td>
<td>0.273</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td>BR-3</td>
<td>NCI-H69</td>
<td>0.120</td>
<td>0.188</td>
<td>0.184</td>
<td>0.068</td>
<td>9.92</td>
</tr>
<tr>
<td></td>
<td>BR-P-EBV</td>
<td>0.089</td>
<td>0.053</td>
<td>0.242</td>
<td>0.013</td>
<td></td>
</tr>
</tbody>
</table>

a EBV lines were fused with KR-4 and selected in HAT medium containing ouabain.
b Hybridoma supernatants were tested on glutaraldehyde-fixed cells in 96-well polystyrene culture plates. Supernatants collected at various times postfusion were tested in an ELISA. Negative controls (KR-4 supernatant, myeloma IgG) gave absorbances of less than 0.25.
c Hybridoma supernatants obtained 65 days postfusion were diluted 1:5 and tested in an ELISA for IgM and IgG content by comparison to affinity-purified standards. The values shown are the mean of duplicate determinations.

from 2 to 50 µg/ml. Several nonspecific hybrids have been cultured for over 1 year, and levels of antibody have remained high (greater than 44 µg/ml). A number of hybrids have been cloned (1 cell/well) and continue to secrete high levels (>30 µg/ml) of tumor-reactive antibody for greater than 5 months. These levels are at least as high, if not higher, than those reported using conventional human × human hybridoma techniques (4, 12, 19).

Despite the recent advancements in human hybridoma technology, several technological difficulties remain. A concern of many investigators using fixed-cell ELISA is the potential deleterious effects of the fixative on cell surface antigens. The recent development of an immunofiltration apparatus permits ELISAs to be performed on live or at least unfixed cells (7). However, extensive use of this system in human hybridoma studies, where large numbers of hybrids must be screened, may be limited by the high cost of the special microtiter plates that are required. Another concern is the use of tumor cell lines as targets for antibody binding. Brenner et al. (1) found that mouse hybridomas secreting antibody reactive with tumor tissue sections failed to react with a corresponding tumor cell line. It has been suggested that certain antigens may be lost or modulated in culture (8). For this reason, it would seem prudent to include immunoperoxidase staining of tumor tissue sections in a screening strategy. Problems may arise due to the predicted high background caused by endogenous human immunoglobulins in the tissue sections. However, passage of the human tumors in nude mice can circumvent this problem (10).

In summary, we have shown that it is feasible to produce human MAbs from lymphocytes of lung cancer patients using our EBV-hybridoma method. The extremely low frequency of B-cell precursors specific for lung tumor cells suggests that only the most efficient human hybridoma systems will be useful. It is probable that with early cloning of hybrids specific anti-tumor antibody production will be stably maintained.

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