Establishment, Molecular Rescue, and Expression of 123AV16–1, a Tumor-reactive Human Monoclonal Antibody

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ABSTRACT

The human monoclonal antibody (mAb) 123AV16–1 was generated by Epstein-Barr virus transformation of peripheral blood lymphocytes from a colorectal cancer patient undergoing active specific immunotherapy with an autologous tumor cell-Bacille Calmette-Guérin vaccine. Direct immunohistochemical staining of tumor and normal pairs of tissues indicated that this human IgA1, A2 mAb preferentially reacted with colon tumor epithelium. To generate a recombinant derivative of this Epstein-Barr virus-transformed cell line, we isolated the expressed complete heavy and light chain genes by a novel strategy and cloned them into modified pSV2-neo and pSV2-gpt expression vectors. The recombinant 123AV16–1 human mAb was expressed in both a murine myeloma and a human-murine heteromyeloma and was secreted as both monomers and dimers. The recombinant 123AV16–1 mAb expressed by both cell lines reacted with human colon tumor xenografts in a manner similar to the mAb derived from the Epstein-Barr virus-transformed cell line, indicating that the antibody specificity was not appreciably altered during the molecular rescue, cloning, or expression.

INTRODUCTION

Human mAbs have many potential advantages over murine, chimeric, and perhaps even humanized antibodies as diagnostic and therapeutic agents in humans. One major advantage of administering human mAbs is the significantly reduced likelihood of inducing an immune response. The HAMA response seen with murine and certain chimeric antibodies (reviewed in Ref. 1) may effectively block the binding of the antibody to its target. HAMA diminishes antibody efficacy of a cancer diagnostic that requires repeated administration and prohibits the multiple injections of large doses necessary for immunotherapy. Administration of repeated doses of a completely human mAb with defined specificity has the greatest potential for effective diagnosis and immunotherapy of tumors without HAMA side effects.

We have utilized PBL from colorectal patients undergoing ASI with autologous tumor cell-Bacille Calmette-Guérin vaccines (2) as a source of human B-cells for producing tumor-reactive antibodies. By immortalizing these human PBL with EBV and screening supernatants for tumor reactive antibodies, we have previously identified two human mAbs, 16.88 (3) and 88BV59 (4), with specific pan-adeno-carcinoma reactivity against colon, breast, ovarian, pancreatic, and prostate tumors. Both human mAbs recognize different epitopes on the cytoplasmic tumor-associated antigen CTAA 16-88, a complex of polypeptides homologous to cytokeratins 8, 18, and 19 (5, 6). Recently, Phase I and II clinical studies for tumor imaging with 16.88 and 88BV59 have shown antibody localization within breast and colon tumors, respectively, suggesting the potential clinical utility of these mAbs for diagnosis and/or therapy of epithelial derived tumors expressing cytokeratin-like antigens (6). Recently, additional human mAbs reactive with tumor-associated antigens distinct from cytokeratin-like antigens have been generated.

In this study, we report the establishment and characterization of the human mAb 123AV16–1 derived by EBV transformation of PBL from a colorectal cancer patient participating in ASI. This is the first report describing a tumor-reactive human IgA1, A2 mAb. To generate a recombinant cell line secreting 123AV16–1, we used the PCR in a novel strategy to rescue and clone the 123AV16–1 heavy and light chain genes. Uniquely constructed expression vectors, derivatives of pSV2-neo and pSV2-gpt (7, 8), were used to successfully express the 123AV16–1 mAb from heavy and light chain cDNAs in both the murine myeloma P3X63Ag8.653 (9) and a proprietary human-murine heteromyeloma HMH. This study contains the first successful expression of a completely human IgA1, A2 recombinant mAb in nonimmunoglobulin-producing myeloma cells.

MATERIALS AND METHODS

Development and Purification of the Human mAb 123AV16–1. The 123AV16–1 human B-cell line was generated by EBV transformation of PBL from a colorectal cancer patient participating in an ASI clinical vaccine trial (2). PBL were obtained 1 week after the second injection of 10E6 X-irradiated autologous tumor cells, admixed with 10E6 colony-forming units of TICE Bacille Calmette Guérin, processed as described previously (10), and immortalized by incubation with EBV containing supernatants from the marmoset lymphoblastoid cell line B95–8 (11). Twenty-five to 50 million PBL were centrifuged at 400 x g for 10 min, resuspended in 5 ml of freshly prepared 4-day-old B95–8 supernatant, and incubated for 90 min at 37°C. PBL were centrifuged at 400 x g for 10 min and resuspended in BRI-2 medium (JRH Biochemicals, Lenexa, KS) containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD) at a density of 5 x 10E6 to 1 x 10E7 cells/well in 96-well plates, along with 5 x 10E5 X-irradiated (20,000 rads) J774 mouse macrophage cells as feeders. Wells containing immunoglobulin-producing cells secreting >2.5 µg/ml of antibody were screened on a panel of human colon xenografts. (12).

123AV16 was selected based on its strong reactivity with this human tumor xenograft panel and was cloned at limiting dilution. A tumor-reactive subclone, 123AV16–1, was identified, expanded, and seeded into hollow fiber culture cartridges. The highly concentrated, hollow fiber-produced mAb was purified by ammonium sulfate precipitation followed by Sephacryl S-300 gel filtration column chromatography (Pharmacia, Piscataway, NJ). Purity was judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be >95%.

Isotyping and Quantitation of Human mAb 123AV16–1. The 123AV16–1 isotype was determined by ELISA using Immulon I microtiter plates (Dynatech, Alexandria, VA) coated at 5 µg/ml with a goat anti-human IgG plus IgA plus IgM (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). Purified human IgG, IgA, or IgM antibodies (OTC-Cappel, West Chester, PA) were used as standards. Supernatants containing 123AV16–1 were added to the wells and incubated at 37°C for 1 h. The plates were washed three times with PBS containing 0.05% Tween 20, followed by the addition of specific HRP-conjugated antibodies to human γ, α, or μ heavy chains (Kirkegaard and Perry Laboratories, Inc.). Following a 1-h incubation at 37°C, the plates were washed and color development was achieved by the addition of...
3.3'-5.5' tetramethyl benzidine (100 μg/ml; Sigma Chemical Co., St. Louis, MO) in 0.1 m sodium acetate buffer (pH 5.5) containing 0.003% (v/v) H2O2. After incubation for 5–10 min at 22°C, the reaction was stopped by the addition of 50 μl 4 M H2SO4, and the absorbance was measured at 450 nm. The light chain class was determined by using an Oschnerolony immunodiffusion kit (The Binding Site, San Diego, CA) following the manufacturer’s instructions.

An IgA capture ELISA was used to quantitate 123AV16-1 levels in supernatants. Goat anti-human IgA was used as the capture reagent along with an HRP-labeled goat anti-human IgA conjugate in the protocol described above.

**Indirect Immunoperoxidase Staining of Human Colon Tumor Xenografts.** Human colon tumor xenografts were propagated in female athymic nude mice (MCr-nu; Frederick Cancer Research Facility) as described previously (13). Animals were maintained according to Food and Drug Administration guidelines in an American Association for Accreditation of Laboratory Animal Care-accredited facility. Five-μm cryostat-cut sections of frozen tissue were air-dried overnight and reacted with 123AV16-1 supernatants for 2 h at 22°C. The sections were washed by immersion in PBS and incubated with peroxidase conjugated goat anti-human IgA for 1 h. Color was developed with DAB (Sigma) for 5–10 min. The sections were counterstained with Harris hematoxylin (VWR Scientific, Bridgeport, NJ), dehydrated in alcohol, cleared, and mounted with Permount. Sections were viewed microscopically for the presence of dark brown staining.

**Doxorubicin Staining of Primary Human Tissues.** Normal and malignant human tissues obtained during surgical procedures or at autopsy within 2 h of death by the National Disease Research Interchange (Philadelphia, PA) were placed in cryomolds (Miles Laboratories, Naperville, IL), covered in ornithine carbamyl transferase (Miles Laboratories, Elkhart, IN), and frozen in liquid nitrogen. 123AV16-1 biotinylation was performed by incubation of the mAb at 1.0 mg/ml with normal human serum-biotin at a 1:75 molar ratio for 4 h at 22°C. Free biotin was removed by passage over a PD-10 column (Pharmacia), and immunoreactivity was determined by titration on a colon tumor xenograft panel.

Five- to seven-μm cryostat-cut sections of frozen tissue were mounted on slides, and endogenous peroxidase was quenched by incubation in 0.3% (v/v) H2O2 in PBS. Non-specific binding of biotin or avidin was blocked using Avid-Biotin blocking reagents (Vector Laboratories, Burlingame, CA) following the manufacturer’s instructions. Sections were blocked for 30 min with normal human IgA at 0.5 μg/ml in Hanks’ balanced salt solution (JRH Biochemicals) containing 2% bovine serum albumin (Sigma). Biotinylated mAb dilutions were incubated with the sections for 2 h at 22°C. Sections were washed with PBS and incubated with avidin-biotin complex peroxidase (ABC Elite Kit PK6100, Vector Laboratories) following the manufacturer’s instructions. Color was developed with DAB as described.

Inhibition of direct immunoperoxidase staining was performed by preincubating the primary human colon tumor sections for 2 h at 22°C with different antibody preparations at concentrations ranging from 0 to 40 μg/ml. Subsequently, the sections were incubated with the biotinylated native 123AV16-1 at 5.0 μg/ml and processed as described previously.

**PCR Cloning and Sequencing.** Fig. 1 depicts the strategy used for “rescueing” the 123AV16-1 heavy and light genes. Total RNA from 1 X 108 tumors was isolated by TRIzol reagent (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer’s instructions. The cDNA was purified by phenol extraction and resuspended in 11 μl of deionized water. An equimolar “mixture” of the 123AV16-1 heavy and light genes. Total RNA from 1 X 108 tumors was isolated by TRIzol reagent (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer’s instructions. The cDNA was purified by phenol extraction and resuspended in 11 μl of deionized water.

The nucleotide sequences of the 123AV16-1 heavy and light chain gene products were determined using the Cycle sequencing kit (Life Technologies, Inc., Gaithersburg, MD) according to manufacturer’s specifications, initially utilizing the original 5'- and 3'- oligonucleotides as primers. Homologous oligonucleotides were designed based on the initial sequence information, and the Vh and Vl sequences were completed. A second PCR amplification from the original cDNA was performed to adapt the 123AV16-1 heavy and light chain cDNAs for cloning into the pBR1-neo and pBR1-gpt expression vectors. The 5' Vh-specific oligonucleotide encoding amino acids 1–5 of the 123AV16-1 Vh contained an anchored SaII site at amino acids –3 and –2 in the leader, along with a serine (TCA) at amino acid –1 (5'-GATGTGCGACTCGAGGTCATTG-3'). The 3' Vl oligonucleotide homologous to carboxyl terminal amino acids 496–491 contained two termination codons and an anchored XhoI site (5'-GATCCTAGATCTACAGGTTGGGCGTCC(A/G)CCTC-3'). The reactions were then performed with 100 ng of each oligonucleotide under the conditions described for the heavy chain amplification. The 1.5-kilobase heavy chain cDNA and 0.75-kilobase light chain cDNA PCR products were digested with SaII and XhoI and directionally cloned into the pBR1-neo and pBR1-gpt expression vectors, respectively.

The polyadenylation signals were added into the constructs for pSV2-neo and pSV2-gpt (7, 8). A murine heavy chain enhancer, Vh promoter, and an intron-containing leader derived from an anti-tumor murine hybridoma3 drive expression of the heavy and light chain cDNAs in each vector. A 155-base pair 3'-untranslated region of the human y3 constant region containing 5' XhoI and 3' NotI restriction sites was inserted into each vector to provide a unique polyadenylation signal and site for immunoglobulin cDNAs (Fig. 1). These chimeric-controlling elements at the 5'- and 3'-ends of the 123AV16-1 heavy and light chain cDNAs facilitate expression of this human mAb in myeloma cells.

**Transfection of 123AV16-1 Light and Heavy Chain cDNA Expression Vectors.** pBR1-gpt-123AV16-1 light chain was transfectd by lipofection into both the murine myeloma P3X63Ag8.653 (9) and the proprietary HMM. Neither of these cell lines synthesize endogenous murine or human heavy or light chains (data not shown). Four million cells were exposed to 10 μg of DNA with lipofectin (Life Technologies, Inc.) according to the manufacturer’s instructions. Cells were plated in 96-well tissue culture plates. After 48 h, BRI-2 media with 10% fetal bovine serum containing 0.75 μg/ml mycophenolic acid (Life Technologies, Inc.), 1.9 μg/ml hypoxanthine (Sigma), and 32.15 μg/ml xanthine (Sigma) was added to the P3X63Ag8.653 cells. HMM cells were selected in media containing 1.5 μg/ml mycophenolic acid. Western blot analysis of cytoplasmic lysates from resistant cells was used to screen for expression of the 123AV16-1 light chain.

HMM and P3X63Ag8.653 cell lines expressing the 123AV16-1 light chain were subsequently transfected with pBRI-neo-123AV16-1 heavy chain DNA by lipofection. Cells harboring light and heavy chain vectors were selected in media supplemented with the appropriate concentrations of mycophenolic acid and 500 μg/ml G418 (Life Technologies, Inc.) according to the manufacturer’s instructions. Cells were plated in 96-well tissue culture plates. After 48 h, BRI-2 media with 10% fetal bovine serum containing 0.75 μg/ml mycophenolic acid (Life Technologies, Inc.), 1.9 μg/ml hypoxanthine (Sigma), and 32.15 μg/ml xanthine (Sigma) was added to the P3X63Ag8.653 cells. HMM cells were selected in media containing 1.5 μg/ml mycophenolic acid. Western blot analysis of cytoplasmic lysates from resistant cells was used to screen for expression of the 123AV16-1 light chain.

**Western Blot Analysis.** Cytoplasmic lysates were prepared from 1 X 106 cells (17), reduced with 0.36 M 2-mercaptoethanol, boiled in 2.0% sodium dodecyl sulfate, and electrophoresed through a Tris-glycine gel. The gel was dried and incubated with the indicated antibodies. The transfer efficiency of the membrane was confirmed by Ponceau S staining.

3 Unpublished data.
ESTABLISHMENT, RESCUE, AND EXPRESSION OF 123AV16-1

FIG. 1. Strategy for rescuing and cloning the 123AV16-1 heavy and light chain cDNAs. pBRI expression vectors are modified pSV2-neo and pSV2-gpt (7, 8) vehicles which drive expression of the human Ig cDNAs from a murine heavy chain promoter and enhancer elements and also contain a murine leader with its intervening sequence. kb, kilobases; L, light; H, heavy.

RESULTS

Characterization of 123AV16-1. The reactivity of purified, biotinylated 123AV16-1 to both tumor and normal human tissues is shown in Table 1. This mAb at 2.5 µg/ml reacted with 54% of primary colon tumors and 37% of colon tumor metastases. In summary, 123AV16-1 reacted moderately to strongly with cytoplasmic elements present in 48% of human colon tumors (28 of 58) tested. The weak reactivity observed at 2.5 µg/ml of 123AV16-1 with 19% of normal colon tissues examined was due to staining of superficial glandular and ductal epithelium. The antibody did not react with breast, gastric, lung, ovarian, pancreatic, or prostatic tumors. 123AV16-1 reacted with 2 of 9 (22%) kidney tumors but not with normal kidney, breast, stomach, lung, ovary, pancreas, or prostate (Table 1). Thus, 123AV16-1 exhibits quantitative specificity for an antigen associated primarily with colon tumors. The EBV-transformed B-cell line secretes 5.5 µg/ml of IgA λ light chain from a 5-day culture (data not shown).

Sequence of the 123AV16-1 VH and VL. PCR was used to rescue the expressed 123AV16-1 heavy and light chain genes from the EBV-transformed human B-cell line as outlined in Fig. 1, and the PCR products were sequenced directly. The VH and VL nucleotide sequences with their deduced amino acid sequences are shown in Fig. 2. The 123AV16-1 VH is a member of the VH3 family and displays the highest degree of homology (89%) to germline VH3 clone 43 (18). While the expressed JH sequence was JH5, the D segment exhibited 40% homology to the DQ23 germline D segment (16). Unique CDR3...
Table 1 Tissue reactivity of 123AV16-1 on malignant and normal human tissues

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Reactivity to tumor (%)</th>
<th>Reactivity to normal (%)</th>
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<tr>
<td>Colon</td>
<td>28/58 (48)</td>
<td>4/21* (19)</td>
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<tr>
<td>Primary tumor</td>
<td>21/59 (54)</td>
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<tr>
<td>Metastatic tumor</td>
<td>7/19 (37)</td>
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<tr>
<td>Kidney</td>
<td>2/9 (22)</td>
<td>0/5 (0)</td>
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<tr>
<td>Breast</td>
<td>0/10 (0)</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>Lung</td>
<td>0/18 (0)</td>
<td>0/7 (0)</td>
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<tr>
<td>Adenocarcinoma</td>
<td>0/5 (0)</td>
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<tr>
<td>Squamous</td>
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<tr>
<td>Small Cell</td>
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<tr>
<td>Melanoma</td>
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<tr>
<td>Ovary</td>
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<td>Prostate</td>
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<tr>
<td>Stomach</td>
<td>0/10 (0)</td>
<td>1/6 (16)</td>
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* Reactivity with normal colon was due to weak staining of glandular and ductal epithelium.

The five nucleotide changes scattered among CDR1 and CDR3 all result in amino acid replacements, suggestive of somatic mutation.

Expression of Recombinant 123AV16-1 Human mAb. pBRgpt-123AV16-1 light was transfected into two nonimmunoglobulin-producing myeloma cell lines, P3X63Ag8.653 (9) and HMH. Transfectants were selected in the presence of mycophenolic acid, and the supernatants were subsequently screened by ELISA for secretion of 123AV16-1 light chain. All mycophenolic acid-resistant clones were negative for light chain secretion, suggesting that the 123AV16-1 light chain remained in the cytoplasm. Fig. 3 shows a representative Western blot of cytoplasmic lysates prepared from two HMH and one P3X63Ag8.653 clone. All clones expressed 123AV16-1 light chain intracytoplasmically at levels ranging from approximately 25 to 75 ng from 10^5 cells. The HMH and P3X63Ag8.653 clones expressing the 75 has created a potential N-linked glycosylation site (Asn X Thr) at amino acid 75 in FR3.

The 123AV16-1 VH is a member of the VA1 germline gene family with 95% homology to both the VA1 DPL5 germline gene (21) and to the HUMIGLAM sequence derived from a B-cell lymphoma patient (22). This chain utilizes the A2 joining region and the A2 constant region class. The expressed VH contains seven sequence differences in its framework regions relative to its presumed germline counterpart, resulting in three amino acid replacements and four silent mutations.

Expression of Recombinant 123AV16-1 Human mAb. pBRgpt-123AV16-1 light was transfected into two nonimmunoglobulin-producing myeloma cell lines, P3X63Ag8.653 (9) and HMH. Transfectants were selected in the presence of mycophenolic acid, and the supernatants were subsequently screened by ELISA for secretion of light chain. All mycophenolic acid-resistant clones were negative for light chain secretion, suggesting that the 123AV16-1 light chain remained in the cytoplasm. Fig. 3 shows a representative Western blot of cytoplasmic lysates prepared from two HMH and one P3X63Ag8.653 clone. All clones expressed 123AV16-1 light chain intracytoplasmically at levels ranging from approximately 25 to 75 ng from 10^5 cells. The HMH and P3X63Ag8.653 clones expressing the

Fig. 2. Nucleotide and deduced amino acid sequences of the human mAb 123AV16-1 VH and VL. (A) 123AV16-1 VH sequence from amino acids 1–113. Underlined amino acids, complementary determining regions CDR1 (31–35a), CDR2 (50–65), and CDR3 (95–102). *, sequence data is under Genbank accession no. L33984. (B) 123AV16-1 VL sequence from amino acids 1–107. Underlined amino acids, complementary determining regions CDR1 (24–34), CDR2 (50–56), CDR3 (89–97). *, sequence data is under Genbank accession no. L33985. The amino acid enumeration for both the 123AV16-1 VH and VL is according to Kabat et al. (16).
Additionally, the specificity of the recombinant 123AV16-1 was examined by testing its ability to inhibit the reactivity of the native 123AV16-1 on four primary human colon carcinomas previously selected from Table 1. Fig. 6 demonstrates that the r123AV16-1 inhibits the biotinylated native 123AV16-1 from immunohistochemically reacting with the four primary human colon tumors. This qualitative inhibition is equal to or slightly better than the inhibition exhibited by the native nonbiotinylated antibody. The slight differences in inhibition may be attributed to the different antibody preparations. The recombinant 123AV16-1 was concentrated 20-fold from 10% serum containing media to achieve a concentration of 40 µg/ml, whereas the native antibody was purified by gel filtration. A different recombinant human mAb, which did not react with human colon tumors, was concentrated in the same fashion and exhibited modest inhibition at concentrations of 20–40 µg/ml (data not shown). The results from Figs. 5 and 6 suggest that the recombinant and native antibodies have similar antigenic specificity.

DISCUSSION

The 123AV16-1 EBV-transformed human B-cell line secretes an IgA1 A2 mAb, which is more restricted in its tumor reactivity than the pan-adenocarcinoma reactivity shown previously for two other human mAbs, 16.88 (IgM, K; Refs. 3 and 24) and 88BV59 (IgG3, K; Ref. 4), which also were generated by EBV transformation of PBL from patients participating in ASI. The human mAb 123AV16-1 does not react with CTAA 16-88 but recognizes another uncharacterized cytoplasmic tumor associated antigen present in human colon tumors. The specificity of 123AV16-1 for colon tumors makes it a potentially useful reagent for the therapy of colorectal cancer. Currently, there is no clinical data regarding the utility of a human IgA1 light chain mAb as a cancer immunodiagnostic or immunotherapeutic agent. We are in a position to administer radiolabeled 123AV16-1 to colorectal cancer patients and to determine its efficacy in the clinic.

We used a novel strategy for molecularly rescuing and cloning the 123AV16-1 heavy and light chain genes (Fig. 1). This strategy, which differs from previous methods (15, 25), utilizes two rounds of PCR in separate reactions to generate heavy and light chain cDNAs that are adapted for cloning directly into the expression vectors, pBRI-neo and pBRI-gpt, respectively. Our initial step uses amplification of immunoglobulin genes using degenerate 5' primers located within the V\textsubscript{H} and V\textsubscript{L} regions.

Because the cognate antigen recognized by 123AV16-1 has not been determined, the specificity of r123AV16-1 in mAb was tested by its ability to react with a panel of four human colon xenografts. Fig. 5 shows a representative indirect immunohistochemical staining of a well-differentiated human colon tumor xenograft, THO (13). The qualitative staining by recombinant 123AV16-1 expressed by both HMH and P3X63Ag8.653 is indistinguishable from each other and from the staining pattern exhibited by 123AV16-1 purified from the EBV-transformed B-cell line. All three antibodies reacted strongly and specifically with colon tumor epithelial cells at 5.0 µg/ml. No reactivity to the underlying connective tissue was noted.

Fig. 3. Western blot of cytoplasmic lysates expressing the r123AV16-1 A light chain. Lanes 1 and 2, recombinant 123AV16-1 A light chain expressed by HMH; Lane 3, recombinant 123AV16-1 A light chain expressed by P3X63Ag8.653; Lane 4, 150 ng of purified human A light chain, Bence Jones protein (Calbiochem, La Jolla, CA). kD, molecular weight in thousands.

Fig. 4. Western blots of supernatants containing recombinant 123AV16-1. Lanes 1 and 8, Sephadryl 300 high molecular weight fraction of 123AV16-1 dimer (Mr, 250,000–340,000) purified from the EBV-transformed human B-cell line; Lanes 2 and 5, recombinant 123AV16-1 from HMH; Lanes 3 and 6, recombinant 123AV16-1 from P3X63Ag8.653; Lanes 4 and 7, 123AV16-1 purified from the EBV-transformed human B-cell line containing dimer (Mr, 340,000), monomer (Mr, 170,000), and HL (Mr, 85,000). Approximately 200 ng of purified 123AV16-1 from concentrated culture supernatants and 200 ng of purified 123AV16-1 from the EBV-transformed human B-cell line were electrophoresed through a 4–12% polyacrylamide gel, blotted, and developed with anti-α-HRP and anti-α-HRP conjugates. kD, molecular weight in thousands.
and V_L leader region along with 3’ constant region primers. PCR amplification utilizing primers within the leader sequence is preferred to cloning within the variable framework regions (15, 25), which could introduce amino acid variations that may compromise the scaffolding responsible for supporting the antigen-binding site (26, 27). A similar approach was utilized by Coloma et al. (28) for expressing a chimeric anti-dansyl antibody in myeloma cells.

From partial sequence analysis of the amino-terminal encoding nucleotides of the initial PCR products, we designed homologous primers encoding the first five amino acids of the VH and VL. The second PCR products generated from the homologous 5’ and 3’ constant region primers for both the heavy and light chain were completely sequenced to ensure that expressible heavy and light chain cDNAs were generated without stop codons. Sequencing directly the second PCR product in its entirety alleviated the necessity of an additional cloning step often used (28, 29, 30) for ease of sequencing. These second PCR products contained appropriate restriction sites and were directionally cloned into the pBRI expression vectors.

The novel cDNA-based pBRI expression vectors were uniquely constructed with a single intervening sequence located within the anti-ricin murine VH leader. Neuberger and Williams (31) have shown that a single intervening sequence between the leader and VH is sufficient for RNA expression of the murine anti-NP μ heavy chain when driven by the VH promoter/lg heavy enhancer combination, and Morrison et al. (28) have demonstrated the requirement of an intervening sequence for κ chain expression when driven by a V_L promoter/lg heavy enhancer combination. Our expression vectors are different from those of Coloma et al. (28), Xiang et al. (32), Queen et al. (33), and Gillies et al. (34), which contain genomic heavy chain constant regions including introns and V_L-CL intervening sequences. To simplify the rescue, cloning and expression of heavy and light chain genes from EBV-transformed human B-cell lines, we incorporated the single murine leader containing intron, complete human heavy and light chain cDNAs generated by the PCR, and appropriate regulatory sequences into the expression vectors.

The production levels of different chimeric antibodies from expression vectors containing multiple intervening sequences have been reported differently by various investigators as μg/ml in tissue culture over a defined time period, μg/ml from spent tissue culture supernatants, or μg/ml from 10^6 cells in 24 h. The levels of expression vary from less than 1 to 5 μg/ml/10^6 cells per 24 h. Other investigators have created cell lines producing hundreds of μg/ml in fermentors utilizing the amplifiable selective agents dihydrofolate reductase or glutamine synthetase in transfected COS, Chinese hamster ovary, or myeloma cells (35, 36, 37). The promotor and enhancer combination, the expressed VH and V_L, the number of intervening sequences, the antibody isotype, and the recipient cell line all differ in various studies. Each of these factors may influence the level of antibody...
secretion. We have successfully expressed a human IgA1, IgG1, IgG3, and a domain-deleted IgG3 in the HMH cell line from the pBRI expression vectors at levels ranging from 2 to 5 µg/ml in tissue culture over 4 days. We are capable of producing g quantities of human mAbs over a 10-week period in hollow fiber culture cartridges from a cell line secreting 5–10 µg/ml in tissue culture, as demonstrated previously for the human B-cell line, 16.88 (10).

Both P3X63Ag8.653 and HMH transfected with the 123AV16-1 light chain do not secrete this A2 light chain but retain it in the cytoplasm (Fig. 3). The lack of murine A2 light chain secretion has been encountered previously and has been attributed to either a single replacement mutation in FR1 at position 15 (38) or in FR3 at position 62 (39). A comparison of the 123AV16-1 Vλ gene with the Vλ1 germline gene (21, 22) revealed that it does not contain any mutations at these comparable positions in the human Vλ1 gene. Notably, transfection of the 123AV16-1 heavy chain into transfectomas expressing the cytoplasmic light chain (Fig. 3, Lanes 2 and 3) resulted in H2L2 secretion. This is similar to the previous studies with the SI07 κ light chain, which was not secreted when expressed in J588L in the absence of a heavy chain but was secreted as part of an H2L2 antibody when complemented with heavy chain synthesis (40).

The recipient cell line chosen for expressing recombinant mAbs can influence the glycosylation of the antibody, particularly its N-linked carbohydrate composition, as demonstrated previously for a chimeric anti-NIP IgA1 expressed in Chinese hamster ovary-K1 cells (41) and chimeric anti-NIP IgG1, 2, 3, and 4 heavy chains expressed in J558L (42). Glycosylation may be an issue to consider for recombinant human mAbs designated for the clinic. Although we have not determined the composition of the N-linked carbohydrates present on the recombinant 123AV16-1, we have preliminary evidence suggesting that the antibody secreted by both HMH and P3X63Ag8.653 contains the unique O-linked galactopyranosides present within the human IgA1 hinge region, based on their ability to bind jacalin-agarose. Similarly, Shin et al. (44) have shown that a chimeric murine anti-dansyl/human IgD secreted by P3X63Ag8.653 is also capable of binding to jacalin-agarose, indicating that the 5–7 potential O-glycosidic linkages present within the IgD hinge region (43) are utilized by the murine myeloma (44). A comparison of the carbohydrate composition of the r123AV16-1 secreted by the murine myeloma and the HMH with the mAb secreted by the EBV-transformed B-cell line under the same growth conditions may indicate which cell line expresses recombinant antibodies with the most "human-like" carbohydrate structures. The oligosaccharide chains added to immunoglobulins may play an important role in the clearance rate, immunogenicity, and biological specific activity of an injected mAb (45).

This study has demonstrated, preliminarily, that the recombinant 123AV16-1 exhibits similar tumor reactivity as the mAb secreted from the EBV-transformed human B-cell line. Both antibodies react equally well with the THO colon tumor xenograft (Fig. 5) and are capable of inhibiting direct immunoperoxidase staining by the native
antibody of four primary human colon tumors (Fig. 6). The fine antigen specificity of both the native and recombinant 123AV16-1 may be determined after the identification and purification of the cognate tumor-associated antigen. The successful development of this recombinant human IgA provides the foundation for developing a 123AV16-1/IgG1 which may present other properties amenable for clinical utility.

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