Human antibodies for immunotherapy development generated via a human B cell hybridoma technology

Jian Li*†, Tao Sai*†, Marc Berger*, Qimin Chao*, Diane Davidson*, Gaurav Deshmukh*, Brian Drozdowski*, Wolfgang Ebel*, Stephen Harley*, Marianne Henry*, Sara Jacob*, Brad Kline*, Ella Lazo*, Frank Rotella*, Eric Routhier*, Kathryn Rudolph*, Jeaneen Sage*, Paul Simon*, Jun Yao*, Yuhong Zhou*, Mani Kavuru‡, Tracey Bonfield§, Mary Jane Thomassen‡, Philip M. Sass*, Nicholas C. Nicolaides*, and Luigi Grasso*¶

*Morphotek, Inc., 210 Welsh Pool Road, Exton, PA 19341; ‡The Brody School of Medicine, East Carolina University, Greenville, NC 27834; and §Cleveland Clinic Foundation, Cleveland, OH 44195

Communicated by Lloyd J. Old, Ludwig Institute for Cancer Research, New York, NY, January 5, 2006 (received for review November 28, 2005)

Current strategies for the production of therapeutic mAbs include the use of mammalian cell systems to recombinantly produce Abs derived from mice bearing human Ig transgenes, humanization of rodent Abs, or phage libraries. Generation of hybridomas secreting human mAbs has been previously reported; however, this approach has not been fully exploited for immunotherapy development. We previously reported the use of transient regulation of cellular DNA mismatch repair processes to enhance traits (e.g., affinity and titers) of mAb-producing cell lines, including hybridomas. We reasoned that this process, named morphogenics, could be used to improve suboptimal hybridoma cells generated by means of *ex vivo* immunization and immortalization of antigen-specific human B cells for therapeutic Ab development. Here we present a platform process that combines hybridoma and morphogenics technologies for the generation of fully human mAbs specific for disease-associated human antigens. We were able to generate hybridoma lines secreting mAbs with high binding specificity and biological activity. One mAb with strong neutralizing activity against human granulocyte–macrophage colony-stimulating factor was identified that is now considered for preclinical development for autoimmune disease indications. Moreover, these hybridoma cells have proven suitable for genetic optimization using the morphogenics process and have shown potential for large-scale manufacturing.

morphogenics | therapeutic antibody

Several disease-associated antigens are currently being targeted using therapeutic mAbs because of their unique pharmacological and safety profiles. Current strategies for the production of therapeutic mAbs include the use of mammalian cell systems (i.e., CHO or NS0 transfectomas) to recombinantly produce mAbs derived from immunization of transgenic mice bearing human Ig genes (xenomice), humanization of rodent mAbs, or through screening of human mAb phage libraries (1). Early development efforts used rodent systems to generate mAbs; however, high immunogenicity prevented their use in indications where prolonged dosing in humans was required. Therapeutic mAbs have more recently evolved into chimeric (rodent variable and human constant regions), humanized (human sequence except for rodent complementarity-determining regions), and fully human Abs to minimize allergic response. Another strategy entails introducing amino acid changes in the Ab sequence to mask rodent epitopes. In some applications, an important aspect of a therapeutic mAb is its ability to elicit immune effector functions, such as Ab-dependent cellular cytotoxicity. Rodent mAbs have been shown to poorly mediate effector functions in humans because of sequence differences in the Fc region; therefore, chimerization or humanization is required to gain optimal pharmacological properties. In addition, mAbs with fully human sequences may still fail to support Ab-dependent cellular cytotoxicity if they are produced in non-human host cells that may alter native glycosylation pattern of mAbs (2).

In view of these facts, an ideal scenario is one where therapeutic Abs are produced by human B cells. In this case, mAbs would be able to exert human effector functions and have very limited immunogenicity because of their native human structure. The generation of hybridoma or Epstein–Barr virus-transformed lymphoblastoid lines derived from human B cells has been previously reported (3–5); however, there is limited information on the characterization of these Abs and the lines with respect to their long-term stability, suitability to manufacturing processes, and the Ab's pharmacological properties (1).

In this report we present a process employing primary human B cells for generating cell lines producing human mAbs. Human B cells are immunized *ex vivo* in the presence of human antigens and then immortalized by means of cell fusion. Alternatively, selected donors are identified whose sera have high immunoreactivity to antigens of interest. Hybrid cells derived from these individuals' B cells are screened for secretion of antigen-specific mAbs. As a result of this effort, we generated mAbs specific to a number of human antigens, including human mesothelin and granulocyte–macrophage colony-stimulating factor (GM-CSF). One mAb showed strong neutralizing activity against human GM-CSF and is now considered for preclinical development for autoimmune disease indications. In addition, we show that hybridoma lines producing these mAbs are suitable for genetic optimization using the morphogenics whole-genome evolution method that we recently described, which is able to improve qualities associated with Ig titers and affinity (6, 19).

Results

Generation of Antigen-Specific Human mAbs. *Ex vivo* immunizations were carried out by using cryopreserved B cells obtained from volunteer subjects (healthy donors) as described in *Materials and Methods*. Alternatively, B cells were obtained from human subjects whose sera contained high titers of mAbs specific to an antigen of interest. The rationale of the latter approach stems from the possibility that some antigen-specific mAbs could result from an abnormal immune response (as in the case of autoimmune patients) or derive from an *in vivo* immune response to tumor, microbial, or vaccine antigens. In this study we obtained lymphocytes from patients affected by pulmonary alveolar proteinosis, a rare lung disorder of unknown etiology characterized by alveolar filling with floccular material. These patients have

Conflict of interest statement: No conflicts declared.

Freely available online through the PNAS open access option.

Abbreviations: PBMC, peripheral blood mononuclear cell; GM-CSF, granulocyte–macrophage colony-stimulating factor; PE, phycoerythrin; MMR, mismatch repair.

†J.L. and T.S. contributed equally to this work.

¶To whom correspondence should be addressed. E-mail: grasso@morphotek.com.

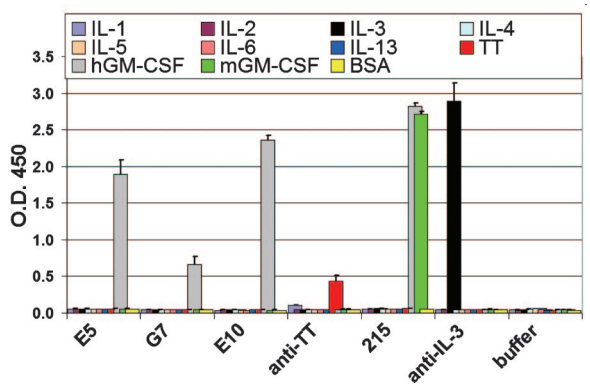

Fig. 1. Antigen panel ELISA for selection of antigen-specific human mAbs. Three GM-CSF-specific human mAbs, E10, G7, and E5, reacted with human GM-CSF and none of the other antigens in the panel. 215, murine mAb specific to human GM-CSF (hGM-CSF); mGM-CSF, murine GM-CSF.

been shown to exhibit high titers of mAbs to human GM-CSF (7). GM-CSF has been identified as a potential factor in the abnormal inflammatory response associated with rheumatoid arthritis as well as other autoimmune diseases (8–10). Therefore, we sought to generate human mAbs against this cytokine that could be applied for prospective immunotherapy. Several antigen-reacting human mAbs were identified after fusion of *ex vivo* immunized cells and from hybridoma libraries generated from pulmonary alveolar proteinosis patients' B cells. Four hybridoma lines, E5 (IgM), G7 (IgM), E10 (IgG), and G9 (IgG), were selected for further studies, and the human mAbs they produce were tested for specificity by ELISA. Fig. 1 shows that E5, G7, and E10 human mAbs reacted only with human GM-CSF and none of the other 10 unrelated antigens tested, including murine GM-CSF, which shares a 53% identity with the human homolog. Similar results were obtained for the G9 hybridoma (data not shown). FACS analyses were carried out to confirm specificity of these human mAbs. Human GM-CSF was allowed to bind to the surface of mouse hybridoma cells, which express membrane-bound mAbs specific to human GM-CSF at a different epitope (data not shown). E5 mAb bound the surface of these cells under these conditions, as indicated by the fluorescence intensity shift (Fig. 2*A Bottom*). This result demonstrates the ability of E5 mAb to bind native human GM-CSF. In absence of cell-bound GM-CSF, E5 mAb did not crossreact with any of the membrane-bound proteins expressed by these hybridoma cells (Fig. 2*A Middle*). Similarly, E10 mAb showed high specificity by FACS analysis (data not shown). In addition, because E10 mAb was found associated to the hybridoma cell membrane, we were able to show its ability to bind soluble, phycoerythrin (PE)-labeled GM-CSF by FACS (Fig. 2*B Middle*). Binding specificity was demonstrated by preincubation of the E10 hybridoma cells with an excess of unlabeled GM-CSF (Fig. 2*B Bottom*).

Human mesothelin is a glycosylphosphatidyl inositol-anchored surface protein expressed in mesothelioma, ovarian, and pancreatic cancer tissues (11, 12). Although its biological role is still unclear, mesothelin has been proposed as a potential target for cell vaccine- and mAb-based therapies of both pancreatic and mesothelioma cancers because of its tumor-restricted expression pattern (13, 14). We sought to develop fully human mAbs against this target by means of *ex vivo* immunization of human B cells. Although several mesothelin-reacting hybridomas were identified after screening, the hybridoma C12 (IgM) was selected for additional testing because of its robust specificity profile (data not shown). This mAb showed strong surface staining of mesothelin-expressing cells but not mesothelin-

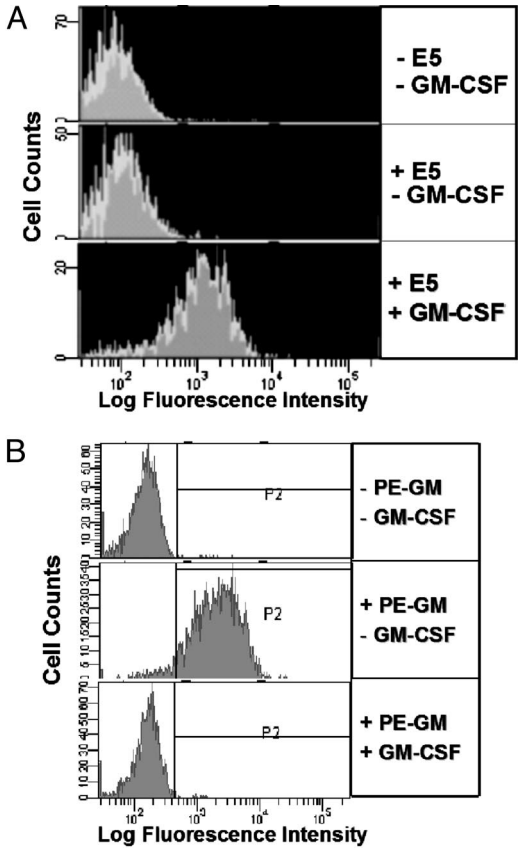

Fig. 2. Human mAbs highly specific to native human GM-CSF. (*A*) Murine hybridoma cells presenting cell surface Ig to human GM-CSF were loaded with (*Bottom*) or without (*Top* and *Middle*) soluble human GM-CSF. mAb E5 was subsequently added (*Middle* and *Bottom*) to the reaction, and its binding to human GM-CSF was measured by using FITC-conjugated goat anti-human Ig. E5 did not bind any of the surface proteins expressed by the murine hybridoma cells (*Middle*) but bound only soluble GM-CSF captured by the cell surface Ig (*Bottom*). (*B*) PE-labeled human GM-CSF (PE-GM) can react to Ig expressed on E10 cell surface (*Middle*). Excess of unlabeled GM-CSF competed for PE-GM binding (*Bottom*). No PE-GM binding was seen in absence of both PE-GM and GM-CSF (*Top*).

negative cells (Fig. 3*A*). No surface staining was observed when normal human Ig from serum was used (Fig. 3*B*).

***De Novo* Class Switch of Human mAbs.** Using the two strategies described above, we were able to generate both IgG and IgM

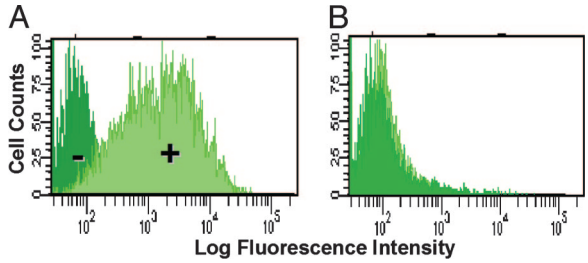

Fig. 3. Human mAb highly specific to human mesothelin tumor antigen. (*A*) mAb C12 (10 μg/ml) was reacted to mesothelin-negative (−) or positive (+) cells followed by FITC-conjugated goat anti-human Ig. Bound Ig causing shifting of fluorescence intensity was detected only with cells expressing mesothelin. (*B*) Normal human IgM was reacted to same cells as in *A*. No fluorescence shift was observed with either cell type, demonstrating specificity of this assay.

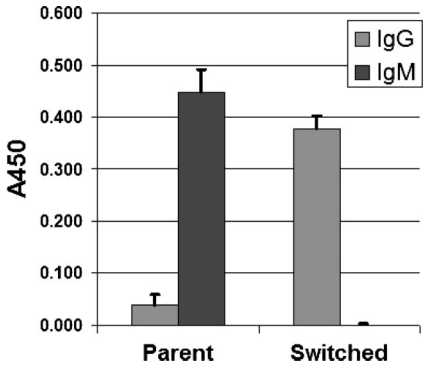

Fig. 4. Class-switched hybridoma cells secrete IgG, retaining antigen binding. Hybridoma E5 cells (Parent) were treated as described in *Materials and Methods*. Hybridoma clones that had class-switched (Switched) were identified by using an ELISPOT-based screening method. An ELISA measuring specific binding to human GM-CSF coated onto plates was carried out to assess binding of either IgM or IgG. Switched IgG mAbs exhibited binding to antigen comparable to that of the parental IgM.

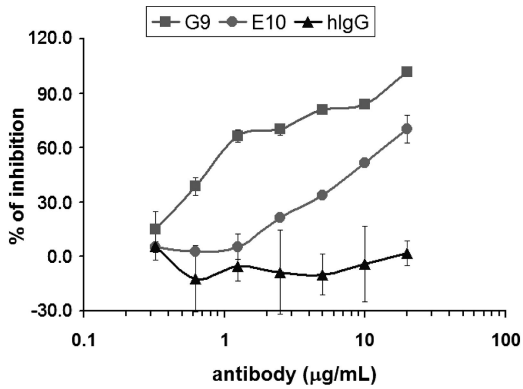

Fig. 5. Biological activity of human mAbs against GM-CSF. A cell-based assay was carried out to determine the level of GM-CSF neutralization (% of inhibition) mediated by G9 and E10 mAbs. Human GM-CSF-dependent TF1 cells were incubated in the presence of 0.1 ng/ml GM-CSF. No inhibition of growth was observed when normal isotype control IgG was included in the reaction, regardless of the concentration used. Both E10 and G9 mAbs neutralized GM-CSF activity.

human mAbs to a variety of human and non-human antigens. On average, one-third of antigen-reacting Abs generated by using the procedure described above are of IgG isotype. Although most of therapeutic Abs in the market are of the IgG isotype, cancer trials testing potentially therapeutic IgM mAbs have shown regression of tumors *in vivo* (15, 16). These clinical responses can be attributable to the ability of IgM to strongly fix and activate the complement pathway and effectively kill tumor cells. IgG binds to the Fc receptors on macrophages and natural killer cells and thus can mediate Ab-dependent cellular cytotoxicity activity against tumor cells. Ideally, both IgG and IgM with identical specificity (same antigen and epitope) should be tested for best pharmacological activity *in vivo*. In the case where an IgG isotype is preferred, we followed a quick robust procedure (see *Materials and Methods*) for *de novo* class-switching of IgM. Using the E5 line as an example, we could readily identify a rare subset of cells that had class-switched to an IgG isotype under the growth conditions used. The E5 IgG showed identical nucleotide sequence in its variable region (data not shown) and reactivity to GM-CSF (Fig. 4) similar to that of the parental E5 IgM. The antimesothelin C12 hybridoma cells were also class-switched to an IgG-secreting hybridoma (data not shown), demonstrating reproducibility of this method.

Biological Activities of Human mAbs. Pharmacological properties sought for therapeutic mAbs that target soluble mediators of disease include the ability to neutralize growth factors. As mentioned above, one such example is GM-CSF as a mediator of rheumatoid arthritis (8–10). We assessed the ability of our human mAbs to block GM-CSF function using a cell-based assay whereby the growth of human erythroblastoid cells (TF1) depends on the presence of this cytokine in their culture medium. As shown in Fig. 5, both E10 and G9 significantly inhibited GM-CSF-dependent cell growth, whereas the human IgG isotype control showed no effect. The difference in potency seen between E10 and G9 correlates well with their apparent affinities of 870 and 14 picomolar, respectively. The E5 mAb showed only minimal neutralizing activity (data not shown), consistent with its lower affinity (5 nM).

Assessment of Titers and Stability of Hybridomas Secreting Human mAbs. An important property of a mAb-manufacturing line is stability of Ig secretion during the entire batch manufacturing cycle. In one scenario, where the cycle duration is ≈2 months, a line doubling every 24 h would go through ≈60 generations from thawing to harvest. We used E5 line as a model for testing mAb titers and production stability of hybridoma generated using our method. A clone derived from this line, 3D2, showed a doubling time of 24 h and was recloned by limiting dilutions after >2 months of continuous culturing. The frequency of producing clones was determined by means of ELISA, measuring Ig concentrations in their conditioned media normalized for cell densities. Fig. 6*A* shows that all E5-3D2 subclones tested secrete high levels of Ig, demonstrating homogeneous retention of Ig production in this cell population after 60 generations. Ig production was then assessed by using a small-scale (15 ml) hollow-fiber system. Cells were inoculated in a hollow-fiber cartridge and continuously fed by using an inline reservoir containing 1 liter of fresh medium. Starting on day 5, all conditioned medium from the cartridge (15 ml) was harvested daily and replaced with fresh medium. Fermentation was carried out for additional 4 days, and daily Ig titers were determined by ELISA by using an Ig standard of known concentration. We recorded a cumulative titer of 1.2 g/liter during the 4-day run. Between days 8 and 9 glucose consumption was at its peak (2 g/liter per day), indicating that cells tolerated well the extremely high cell densities. Production performance was also evaluated

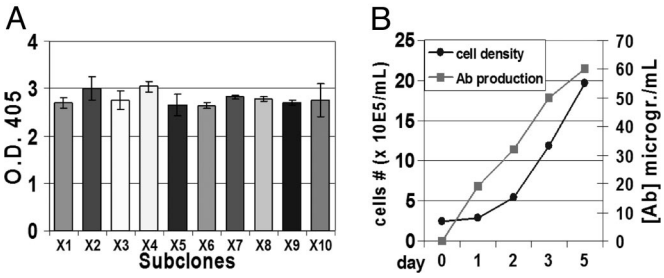

Fig. 6. Assessment of titers and stability of hybridomas secreting human mAbs. (*A*) The hybridoma E5-3D2 line was grown for 60 generations, and then stability of production was assessed by analyzing frequency of producing cells. Subclones (X1–X10) derived from 3D2 cells by means of limiting dilution were randomly chosen, and their Ig production was measured by using an ELISA-based assay. Absorbance at 405 nm was normalized for colony size by visual inspection of the cell-containing wells. (*B*) 3D2 cells were inoculated in a stirred bioreactor containing 1 liter of serum-free medium, and Ig production and number of viable cells were recorded on days 1–5. The specific productivity measured during the log phase was 24 pg per cell per day.

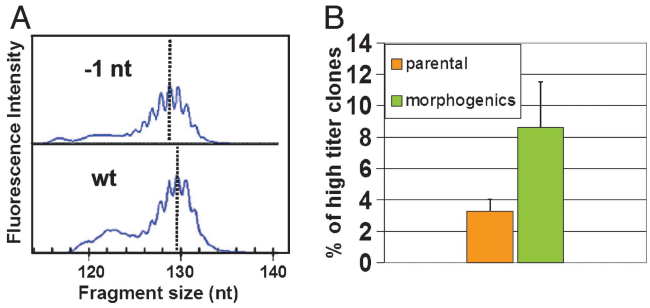

Fig. 7. Genetic optimization of hybridoma-secreting human mAbs by MMR regulation. (*A*) Example of a single-nucleotide deletion in the BAT marker found in E5 hybridoma cells treated with morphogenics. Dotted lines crossing the central peak in the histogram represent the size of wild-type (wt) or contracted (−1 nt) fragment. (*B*) Parental and morphogenics-treated cells were seeded in microplates to yield 3,763 and 2,437 Ig-secreting clones (OD > 0.2), respectively. Ig concentrations were determined by ELISA, and the frequency of clones with OD values >1 was recorded and is expressed as percentage of total number of clones screened.

in a 1-liter-scale fed-batch run by using a stirred bioreactor system. Cells from a frozen ampule were first thawed and inoculated in a shake flask and later seeded in stirred bioreactor (Bauer) containing 1 liter of serum-free medium. Fermentation was carried out until cell viability dropped below 60% (day 6). Ig production and cell densities were recorded between days 1 and 5 and are shown in Fig. 6*B*. During the log phase (days 1–4) we measured a specific productivity of 24 pg per cell per day with a doubling time averaging 23.4 h, suggesting good scalability of these cells from flask to bioreactor while maintaining higher titers.

Genetic Optimization of Hybridoma-Secreting Human mAbs by Means of Mismatch Repair (MMR) Regulation. We previously demonstrated the usefulness of improving the quality of mAb-producing cell lines using a process, termed morphogenics, that entails the transient regulation of MMR (17, 18). After increasing the genetic diversity of the cell pool using this method, high-throughput screenings are typically carried out to identify subclones exhibiting higher titer, affinity (6, 19), or enhanced growth rates (L.G., unpublished observation). E5 cells were subjected to morphogenics to demonstrate the ability to increase phenotypic diversity in the mAb-secreting lines generated by using our hybridoma strategy. MMR inhibition was monitored by detecting microsatellite instability in the BAT polyA repeat marker. Of the 24 BAT alleles analyzed in cells exposed to the morphogenics process, 3 alleles showed alterations that included single-nucleotide deletions (shown in Fig. 7*A*) and insertions. No microsatellite instability was detected in any of the 24 BAT alleles in parental cells. Subsequently, parental or morphogenics-treated cells were seeded by limiting dilutions in microplates. Cell clones were allowed to secret mAbs for 1 week, and their conditioned medium was analyzed for Ig concentrations by ELISA. The frequency of clones with OD > 1 (high Ig secretion) was determined from the total number of clones screened (3,763 for parental and 2,437 for morphogenics pool) and found to have increased by 260% ($P = 0.0014$) in the morphogenics-treated population (Fig. 7*B*). We are currently using this process to enhance growth properties and titers of other hybridoma lines for preclinical development.

Discussion

Here we present a viable strategy for developing human mAbs for immunotherapies using an optimized *ex vivo* immunization and human B cell immortalization process combined with the morphogenics process. Specific human mAbs could be obtained as well by immortalization of B cells from donors exhibiting high serum reactivity to target antigen. With this approach we are able to generate highly specific and biologically active mAbs secreted by stable hybridoma lines. During the preparation of this article we generated specific fully human mAbs against *Staphylococcus* enterotoxin B (unpublished observations), which could potentially be used for anti-bioterrorism strategies. To date, we have succeeded in generating specific human mAbs for all of the antigens targeted. When using human B cells for developing therapeutic mAbs against self-antigens employing a hybridoma-based method, some challenges may arise. First, immune tolerance could prevent identification of human B cells producing mAbs against self-antigens. This has not been our experience or that of other investigators, who have also reported the identification of mAbs derived from peripheral blood mononuclear cells (PBMCs) of normal donors reacting against self-antigens (20, 21). Second, one can predict that, because of lack of sufficient maturation outside of the germinal centers, the binding affinities of mAbs derived by means of *ex vivo* immunization of human B cells is less vigorous than those of mAbs obtained through *in vivo* immunizations. In our *ex vivo* immunizations, the B cells were derived from healthy volunteers who exhibited no measurable serum immunoreactivity to target antigen (titers < 1:100; data not shown); however, after antigen stimulation and B cell immortalization we were able to derive stable hybridomas secreting high-affinity mAbs specific to the target antigen. Additional affinity enhancement could potentially be achieved by means of morphogenics, as we previously reported (6, 19). Third, human B cells used for the generation of mAbs designed for administration to humans may represent a potential vehicle of viral transmission. As a standard operating procedure, we typically prescreen fusion partner cells and PBMCs from healthy donors to confirm absence of viral DNA by PCR, including immunodeficiency 1 and 2, hepatitis B and C, cytomegalovirus, herpesvirus 6, and Epstein–Barr virus (data not shown). As expected, the hybridoma lines we developed to date are negative for this panel of viruses, suggesting that our method does not pose safety risks related to viral transmission more than conventional methods. Last, transfectoma lines are often chosen over hybridoma lines because of their generally better production titers and stability profiles. In our experience, we were able to obtain stable mAb production for >60 doublings and produce >1 g of mAb per liter during a 4-day hollow-fiber fermentation run, suggesting that hybridoma cells generated by using our method are suited for perfusion systems and potentially large-scale manufacturing. Moreover, hybridomas generated by this process have performed well in fed-batch bioreactor runs, suggesting a potential use of these lines for commercial applications. Here we also show that morphogenics can increase phenotypic diversity of hybridoma lines obtained by using our method, whereas previously we used this process to stably (>50 doublings) increase severalfold titers of human Abs secreted by hybridoma lines obtained by other methods (L.G., unpublished observation). In summary, the platform process presented here offers an alternative approach for a rapid and cost-effective development of good-quality, fully human Abs for immunotherapeutic use.

Materials and Methods

Human B Cells, *ex Vivo* Immunization, and Cell Culturing. In all procedures followed, cells were grown in 5% CO_2 at 37°C. Leukopacks were obtained from tetanus toxoid-vaccinated healthy individuals. PBMCs were purified by Ficoll-Paque (Amersham Pharmacia Biosciences), and CD19-positive B cells and CD4-positive T cells were isolated from PBMCs by an EasySep human CD4 and CD19 selection kit (StemCell Technologies), respectively, and mixed to make a B cell/T cell pool (BT4 cells).

BT4 cells were cultured in complete RPMI medium 1640 (Invitrogen), which contained 10% heat-inactivated human serum AB (Nabi), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 55 μM 2-mercaptoethanol (Invitrogen).

For *ex vivo* immunizations, BT4 cells were cocultured in the presence of T and B cell epitopes. Briefly, BT4 cells were seeded at a density of 10^6 per ml in complete RPMI medium 1640 containing 1 unit per ml tetanus toxoid (Cylex) in the presence of irradiated autologous PBMCs at a 1:1 ratio to produce activated T cells (T pool). To generate antigen-activated B cells (B pool), BT4 cells were seeded at a density of 3×10^6 cells per ml in complete RPMI medium 1640 containing 10% human serum AB, 5% of condition medium from activated T cells, 20 units/ml IL-2, 0.5 ng/ml IL-6, 100 units/ml IL-10 (PeproTech), and 250 ng/ml of a mixture of peptides (Bio-World) representing various regions of the targeted antigen, synthesized to contain both T and B cell epitopes as described in ref. 5.

The selected B cell epitopes for GM-CSF were EHVNAIQEARRLLNL, STQPWEHVNAIQEAR, and MASHYKQHCPPTPET. The selected B cell epitopes for mesothelin were EVEKTACPSGKKARE, NKGHEMSPQVATLID, RFVKGRGQLDKDTLD, HVEGLKAEERHRPVR, AVRPQDLDTCDPRQLD, and SFLGGAPTEDLKALSQ.

T and B pools were separately cultured for 7 days and then cocultured (10^6 cells per ml) at a 1:10 ratio, respectively, on a monolayer of irradiated CHO feeder cells in complete RPMI medium 1640 containing 10% heat-inactivated human AB serum and 400 units/ml IL-4 (PeproTech). After 5 days, cocultured T and B pools were fused to generate hybridomas as described.

For the immortalization of pulmonary alveolar proteinosis patients' B cells, 100 ml of whole blood was processed to purify PBMCs. Lymphocytes were cultured for 7–10 days in complete RPMI medium 1640 containing 10% heat-inactivated FBS (JRH Biosciences), 2 ng/ml IL-4 (PeproTech), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 55 μM 2-mercaptoethanol (Invitrogen), 50 μg/ml transferrin, 5 ng/ml phorbol myristate acetate, and 0.5 μg/ml cyclosporine A (Sigma) in the presence of irradiated CHO feeder cells. Lymphocytes were then electrofused as described.

Cell Fusion and ELISA Screening of Antigen-Reacting Hybridomas. Lymphocytes were fused with K6H6/B5 cells (American Type Culture Collection) by using the Cyto Pulse CEEF-50 apparatus (Cyto Pulse Sciences) at a 1:1 lymphocytes:K6H6/B5 ratio. Settings were as follows: the first cycle consisted of alignment (40 V, 15 seconds) and pulse (5 V, 0.1 milliseconds); the second cycle consisted of alignment (75 V, 15 seconds), pulse (800 V, 0.04 milliseconds), and postpulse (60 V, 30 seconds). Fused cells from each immunization experiment were seeded in 20 flat-bottom 96-well microplates at ≈5,000 cells per well in complete RPMI medium 1640 containing 10% heat-inactivated FBS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 55 μM 2-mercaptoethanol (Invitrogen), 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (HAT; Sigma). Medium was replaced weekly, and HAT selection was maintained until completion of antigen reactivity screening (3–5 weeks). On average, 60% of seeded wells contained viable hybridoma cells, and 1,200 clones were screened after each immunization. For the identification of antigen-reacting mAbs, ELISA-based screenings were performed robotically by using a Biomek FX liquid handling system (Beckman) integrated with plate washer and spectrophotometer. Briefly, microtiter plates were coated at room temperature for 6 h with 50 μl per well of in-house produced recombinant antigens (1 μg/ml GM-CSF or 200 ng/ml mesothelin) diluted in coating buffer (50 mM carbonate-bicarbonate, pH 9.4). Plates were then blocked with binding buffer [PBS containing 1% BSA (Sigma) and 0.05% Tween 20 (Bio-Rad)] for 2 h at room temperature. Plates were washed once with washing buffer (PBS containing 0.05% Tween 20), and 50 μl of hybridoma supernatant per well was transferred into the ELISA plates. Binding reaction was carried out at room temperature for 2 h. Subsequently, plates were washed four times, and 100 μl of horseradish peroxidase-conjugated goat anti-human IgG+M (Jackson ImmunoResearch) diluted 1:10,000 in binding buffer was added, and reactions were carried out at room temperature for 1.5 h. Finally, plates were washed four times, and 100 μl of SureBlue substrate (Kirkegaard & Perry Laboratories) per well was added for 10 min. Reactions were stopped by adding 50 μl of 1 N sulfuric acid per well, and the absorbance was determined at 450 nm. An average of 1.5% of screened clones typically reacted to the target antigen as determined by ELISA by using tetanus toxoid as an unrelated antigen.

FACS Analyses. Ig binding and cell washing steps were conducted by using ice-cold binding buffer (DPBS without calcium or magnesium/0.5% BSA), reactions were set up in V-bottom microplates, and samples were analyzed by using a FACSAria apparatus (BD Biosciences). For the FACS experiment shown in Fig. 3*A*, 10^6 murine anti-GM-CSF hybridoma cells (Mul19/2, obtained from Ludwig Institute for Cancer Research) were loaded with 100 ng of GM-CSF per reaction and then incubated with anti-GM-CSF human mAb E5. The binding of GM-CSF-specific mAbs were detected with 10 μg/ml FITC-labeled goat anti-human Ig (Southern Biotechnology Associates). For the FACS experiment shown in Fig. 3*B*, the E10 hybridoma cells were washed and seeded at 500,000 cells per well in a volume of 90 μl. Ten microliters of PE-labeled GM-CSF (R & D Systems) was then added in each well, and cells were incubated on ice for 1 h. For unlabeled GM-CSF competition, the hybridoma cells were preincubated with 5 μg/ml recombinant human GM-CSF (PeproTech) at room temperature for 1 h, washed three times, and then incubated with PE-GM-CSF as above before analysis. For the FACS experiment shown in Fig. 4, A431 and A431-K5 cells (a kind gift of Ira Pastan, National Cancer Institute, Bethesda) were stained with 10 μg/ml C12 mAb or normal human IgM (Jackson ImmunoResearch) diluted in binding buffer, and reactions were carried out as above.

GM-CSF Neutralization Bioassay. The GM-CSF-dependent human erythroleukemia cell line TF1 (American Type Culture Collection) was grown in complete RPMI medium 1640 (see above) containing 10 ng/ml recombinant human GM-CSF (PeproTech). On the day preceding the experiment, TF1 cells were grown in 0.1% BSA in the absence of GM-CSF. Starved cells were washed twice, resuspended in assay medium, and seeded in 96-well microplates at a concentration of 10,000 cells per well. Wells contained assay medium, 100 pg/ml GM-CSF, or GM-CSF preincubated for 1 h with test or isotype control Ig at concentrations indicated in the figure legends. After 3 days, 40 μl of Cell Titer reagent (Promega) was added to each well, and plates were further incubated at 37°C for 1 h. OD was measured at 490 nm in a spectrophotometer, and medium background was subtracted to all samples. Percentage of GM-CSF neutralization was calculated as follows: 100 − (OD with Ig/OD without Ig × 100).

Ab Class Switch. Hybridoma cells were washed once with 10 ml of PBS, resuspended in complete RPMI medium 1640, seeded into flat-bottom 96-well microplates, and incubated at 37°C in 5% CO_2. After 4 days, cells were resuspended by pipetting, and 100 μl was transferred to 20 ELISPOT plates (Millipore) coated with 2.3 μg/ml goat anti-human IgG (H+L) (Jackson ImmunoResearch). The remaining cells in the tissue plates were fed with

an additional 100 μl of complete RPMI medium 1640. After overnight incubation, ELISPOT plates were washed three times with PBS containing 0.05% Tween (PBST), then 100 μl of 2 μg/ml goat anti-human IgG (H+L)-horseradish peroxidase was added, and the plates were incubated for 1 h at room temperature with shaking. Plates were washed three times with PBST, and then 100 μl of 3-amino-9-ethylcarbazole substrate solution (Sigma) was added to wells and incubated for 90 min at room temperature with shaking. Substrate was aspirated, and plates were washed with dH_2O and allowed to air dry. Clones from wells exhibiting positive spots (indicating IgG production) were expanded. The above step was sequentially repeated by reseeding positive clones at 1,000, 100, 10, and 0.25 cells per well while tracking positive wells and until a single-cell colony was identified that secreted IgG.

Fermentation Using Hollow Fibers and Stirred Bioreactor. Cells were seeded at 2.5×10^5 per ml in a 2-liter bioreactor (B. Braun Biostat B-DCU) containing 1 liter of HyQCDM4NS0 serum-free medium (HyClone) maintaining glucose and glutamine at 6 g/liter and 4 mM, respectively. Controlled set points were pH 7.1, dO_2 40% saturation with air, temperature 37°C, and agitation rates at 80 rpm. Two milliliters of sample was harvested daily, 1 ml for cell counting by using a Cedex apparatus and 1 ml used to measure Ig concentrations by ELISA. For the hollow-fiber run, 10^8 viable cells were seeded in a FiberCell system (Bellco) containing 15 ml of complete RPMI medium 1640 and refed by using an inline reservoir containing 1 liter of fresh medium when 50% of the glucose was consumed.

MMR Inhibition (Morphogenics) to Increase Genetic Diversity of Hybridoma Lines. Hybridoma cells were grown in complete RPMI medium 1640 (negative control) or complete RPMI medium 1640 containing 250 μM or 500 μM MMR-inhibiting compound morphocene. Cells were passed at a 1:5 dilution every 3 to 4 days in fresh media with or without morphocene, and after 3 weeks cells were harvested and resuspended at 2×10^6 cells per ml in FACS buffer (PBS with 1% BSA). Cells were stained with 10 μg/ml FITC-conjugated goat anti-human Ig (Jackson ImmunoResearch) for 30 min on ice. Cells were washed with 10 ml of ice-cold FACS buffer and resuspended in 3 ml of FACS buffer. Ten microliters of Viaprobe (Becton Dickinson) was added for 5 min on ice, and viable cells were sorted for high Ig surface staining on a FACSAria cell sorter (Becton Dickinson). The gate was set to sort cells representing the 5% subpopulation with the highest Ig surface staining. For selection of clones with enhanced titers, FACS-sorted cells were seeded in U-bottom 96-well plates and incubated for 1 week at 37°C in 5% CO_2. Fifty microliters of supernatants was harvested from wells and analyzed for IgM production with ELISA by using goat anti-human IgM+G-coated plates. As an internal control, three wells of each ELISA plate were seeded with 50 μl of 10 ng/ml human IgM (Jackson ImmunoResearch). OD values obtained at 450 nm were normalized to the mean values of internal control wells. Wells exhibiting high IgM signals were expanded for further analysis. For microsatellite instability analysis, DNA was extracted from parental or morphocene-treated cells by using the DNeasy Tissue kit (Qiagen). The BAT poly(A) repeat marker (22) was amplified by using the D4 fluorescent-labeled BAT-26-F (5′-tcaccatccattgcacagtt-3′) and BAT-26-R (5′-ctgcgagaaggtactcaccc-3′) primers, pfuUltrahigh-fidelity polymerase (Stratagene), and reactions incubated as follows: 5 min at 95°C; 9 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C, with the annealing temperature decreasing by 1°C each cycle; 30 cycles of 1 min at 94°C, 1 min at 52°C, and 2 min at 72°C; final extension of 10 min at 72°C. Single copy of the marker allele were obtained by using a dilution of DNA the yielded a PCR product in only 50% of the PCRs. PCR products were diluted 1:10 with CEQ sample load solution and then loaded into the Beckman CEQ 8000 Genetic Analysis System for fragment analysis.

1. van Dijk, M. A. & van de Winkel, J. G. (2001) *Curr. Opin. Chem. Biol.* **5,** 368–374.
2. Shinkawa, T., Nakamura, K., Yamane, N., Shoji-Hosaka, E., Kanda, Y., Sakurada, M., Uchida, K., Anazawa, H., Satoh, M., Yamasaki, M., *et al*. (2003) *J. Biol. Chem.* **278,** 3466–3473.
3. Kirman, I., Kalantarov, G. F., Lobel, L. I., Hibshoosh, H., Estabrook, A., Canfield, R. & Trakht, I. (2002) *Hybrid. Hybridomics* **21,** 405–414.
4. Boerner, P., Lafond, R., Lu, W. Z., Brams, P. & Royston, I. (1991) *J. Immunol.* **147,** 86–95.
5. Zafiropoulos, A., Andersson, E., Krambovitis, E. & Borrebaeck, C. A. (1997) *J. Immunol. Methods* **200,** 181–190.
6. Grasso, L., Kline, J. B., Chao, Q., Routhier, E. L., Ebel, W., Sass, P. M. & Nicolaides, N. C. (2004) *Bioprocess Int.* **2,** 58–64.
7. Bonfield, T. L., Kavuru, M. S. & Thomassen, M. J. (2002) *Clin. Immunol.* **105,** 342–350.
8. Campbell, I. K., Bendele, A., Smith, D. A. & Hamilton, J. A. (1997) *Ann. Rheum. Dis.* **56,** 364–368.
9. Cook, A. D., Braine, E. L., Campbell, I. K., Rich, M. J., Hamilton, J. A., Bischof, R. J., Dunn, A. R. & Grail, D. (2001) *Arthritis Res.* **3,** 293–298.
10. Campbell, I. K., Rich, M. J., Bischof, R. J., Dunn, A. R., Grail, D. & Hamilton, J. A. (1998) *J. Immunol.* **161,** 3639–3644.
11. Chang, K. & Pastan, I. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 136–140.
12. Argani, P., Iacobuzio-Donahue, C., Ryu, B., Rosty, C., Goggins, M., Wilentz, R. E., Murugesan, S. R., Leach, S. D., Jaffee, E., Yeo, C. J., *et al*. (2001) *Clin. Cancer Res.* **7,** 3862–3868.
13. Hassan, R., Bera, T. & Pastan, I. (2004) *Clin. Cancer Res.* **10,** 3937–3942.
14. Thomas, A. M., Santarsiero, L. M., Lutz, E. R., Armstrong, T. D., Chen, Y. C., Huang, L. Q., Laheru, D. A., Goggins, M., Hruban, R. H. & Jaffee, E. M. (2004) *J. Exp. Med.* **200,** 297–306.
15. Irie, R. F. & Morton, D. L. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 8694–8698.
16. Irie, R. F., Ollila, D. W., O'Day, S. & Morton, D. L. (2004) *Cancer Immunol. Immunother.* **53,** 110–117.
17. Nicolaides, N. C., Carter, K. C., Shell, B. K., Papadopoulos, N., Vogelstein, B. & Kinzler, K. W. (1995) *Genomics* **30,** 195–206.
18. Nicolaides, N. C., Littman, S. J., Modrich, P., Kinzler, K. W. & Vogelstein, B. (1998) *Mol. Cell. Biol.* **18,** 1635–1641.
19. Nicolaides, N. C., Ebel, W., Kline, J. B., Chao, Q., Routhier, E. L., Sass, P. M. & Grasso, L. (2005) *Ann. N.Y. Acad. Sci.* **1059,** 1–11.
20. Tao, T. W., Leu, S. L. & Kriss, J. P. (1985) *J. Clin. Endocrinol. Metab.* **60,** 279–282.
21. Baritaki, S., Zafiropoulos, A., Georgopoulos, E., Souris, S. & Krambovitis, E. (2001) *Cancer Immunol. Immunother.* **50,** 109–114.
22. Blake, C., Tsao, J. L., Wu, A. & Shibata, D. (2001) *Am. J. Pathol.* **158,** 1867–1870.