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Construction and testing of mouse–human heteromyelomas for human monoclonal antibody production

(dominant selection markers/DNA-mediated gene transfer/interspecies somatic cell hybrids/human monoclonal antibodies/Epstein–Barr virus)

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ABSTRACT **FU-266, a mutant human myeloma cell line sensitive to hypoxanthine/aminopterin/thymidine (HAT), was transfected by protoplast fusion with DNA of the recombinant plasmid vector pSV2-*neo*^{R}, thus acquiring a dominant marker conferring resistance to the antibiotic G-418. One of the resultant *neo*^{R} clones, E-1, was fused to irradiated (500 rads) or unirradiated cells of the HAT-sensitive, G-418-sensitive, nonproducer mouse myeloma line X63-Ag8.653. Hybrid clones were selected in G-418 plus ouabain, thus preserving their HAT sensitivity. Small numbers of human chromosomes were retained in all such hybrids, but most of them ceased secreting human myeloma IgE(λ). Selected hybrid clones were then tested as malignant fusion partners in a series of fusions with polyclonally activated human B lymphocytes and with antigen-primed human B lymphocytes, in some instances after transformation of the latter with Epstein–Barr virus. The yield of viable chimeric hybridomas has been consistently high, as has the proportion of hybridomas secreting human immunoglobulin molecules unpermuted with mouse or human myeloma heavy or light chains. Secretion by many subcloned hybridomas has been stable for over 6 months, and several antigen-specific human monoclonal antibodies have been generated. Thus these heteromyeloma cell lines appear to have significant advantages for human monoclonal antibody production.**

The development by Köhler and Milstein (1, 2) of the hybridoma procedure for mouse monoclonal antibody production has opened a new era in immunology. The fact that the cell lines thus derived are cloned and immortal assures the monoclonality and permanent availability of their antibody products, and antibody yield is limited only by cell culture volume. However, clinical use of xenoantibodies in human patients is likely to be severely limited by the fact that they will be treated as foreign proteins by the human immune system. Thus, for diagnostic and therapeutic applications in man, the production of human rather than mouse or other rodent monoclonal antibodies would clearly be desirable.

Early attempts to generate immortalized human immunoglobulin-producing cells involved the fushion of human lymphoid cells with mouse myeloma cells to create chimeric hybridomas (3–9). However, with rare exceptions (6–8), such mouse–human hybridomas have tended to cease human immunoglobulin production due to the selective loss of human chromosomes (10, 11), or to disturbances of gene expression (12). Transformation of antigen-primed human B lymphocytes with the Epstein–Barr virus (EBV) (13–20) has had some success, but such cultures usually secrete low levels of antibody and have tended to cease antibody production entirely after a variable period (13). These problems have also been encountered in most, though not all, of the fusions of antigen-primed human B lymphocytes with EBV-transformed B lymphoblastoid cell lines (LCL) (8, 21–28). Moreover, such hybridomas usually secrete permuted immunoglobulin molecules derived from both fusion partners. Some increase in secretion level and an improvement in stability was achieved by combining EBV transformation with fusion with a LCL in the "EBV-hybridoma" technique (29, 30).

About three years ago, our group (31) succeeded in fusing a mutant human myeloma cell line with antigen-primed human B lymphocytes to yield human–human hybridomas secreting monoclonal antibodies of predefined antigenic specificity. Although others (8) have now confirmed this result, the yield of viable hybrids with this mutant human myeloma is too low for practical application, probably because, like the few other human myeloma cell lines extant, it is near-diploid and relatively slow growing, and its hybrids are therefore vulnerable to suppression of the malignant phenotype (32, 33). Accordingly, there is an urgent need for the development of new cell lines capable of generating consistently high yields of viable hybridomas and high, sustained levels of human monoclonal antibody production after fusion with antigen-primed human B lymphocytes. In this paper, we report the construction and testing of mouse–human hybrid myeloma ("heteromyeloma") cell lines that appear to have favorable characteristics as malignant fusion partners for human monoclonal antibody production.

MATERIALS AND METHODS

Selection of Hypoxanthine/Aminopterin/Thymidine (HAT)-Sensitive Mutant Human Myeloma Cell Line. FU-266, a HAT-sensitive mutant of the U-266 human myeloma cell line (34), was derived by selection in 6-thioguanine (20 μg/ml) after first freeing the parental myeloma cells (kindly provided by Kenneth Nilsson, Uppsala, Sweden) of mycoplasma infection by heat treatment at 41°C for 48 hr (35). The 6-thioguanine-resistant cells were shown to be HAT sensitive; unlike an earlier mutant, U-266 AR$_1$ (SKO-007), derived by selection in 8-azaguanine (31), they revealed no detectable leakiness or reversion. Like the parental U-266 cell line, FU-266 cells have a modal number of 44 chromosomes, secrete IgE(λ), and have a doubling time of 40–50 hr. Their capacity to act as malignant fusion partners in fusions with antigen-primed human B lymphocytes is similar to that of the previously described SKO-007 mutant (31).

Insertion of Dominant Selection Marker. FU-266 cells were transfected with DNA carrying a bacterial gene for neomycin

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Abbreviations: EBV, Epstein–Barr virus; LCL, lymphoblastoid cell line; HAT, hypoxanthine/aminopterin/thymidine; PWM, pokeweed mitogen; PBL, peripheral blood lymphocytes.

resistance (*neo*^{R}) by using the recombinant plasmid vector pSV2-*neo*^{R} (36). To introduce this vector into the myeloma cells, we chose the protoplast fusion technique (37, 38), modified for use with lymphoid cells as will be described elsewhere. The transfected myeloma cells were then incubated in the presence of G-418, a 2-deoxystreptamine antibiotic structurally related to gentamycin that kills mammalian cells by blocking protein synthesis.‡ Growth occurred in many culture wells; cells from several of these cultures were cloned and expanded. Hybridization with radiolabeled simian virus 40 probe verified the presence of the transfected genomes in high molecular weight cellular DNA. The fastest growing clone, designated E-1, showed no detectable alteration in its biological properties with respect to HAT sensitivity, IgE(λ) secretion, growth rate, or capacity to yield human–human hybridomas.

Generation of Heteromyeloma Cell Lines. The X63-Ag8.653 HAT-sensitive mouse myeloma cell line (39) was selected as the murine malignant fusion partner because of its rapid growth rate, excellent fusion characteristics, and loss of the capacity to secrete mouse immunoglobulin heavy or light chains. This cell line was incubated in the presence of G-418 and found to be highly sensitive, with no survivors after approximately 10 days. Two sets of fusions of FU-266 *neo*^{R} (clone E-1) with X63-Ag8.653 cells were carried out as described below. Since human cells are about 1,000-fold more sensitive to ouabain than are mouse cells (40), selection of the fused heteromyelomas was then carried out in the presence of 0.5 μM ouabain plus G-418 at 400 μg/ml. In the first series of fusions, in which intact, unirradiated mouse myeloma cells were used, approximately 30 hybrid clones were obtained, of which a small number were selected for further study. The second series of fusions was carried out after exposing the mouse myeloma cells to a single dose [500 rads (5 grays)] of ^{137}Cs γ rays, which was intended to damage some of the murine chromosomes (41). Selection was again carried out in the presence of ouabain plus G-418. Many clones were again obtained, of which a small number were selected for further study.

Fusion Procedure. Cell fusion was carried out according to Oi and Herzenberg (42), modified as follows. Myeloma or heteromyeloma cells (1×10^7) and lymphocytes (4×10^7) were each washed twice with 50 ml of Ca- and Mg-free phosphate-buffered saline (PBS-CMF) (43). The cells were then mixed, washed with 10 ml of the phosphate-buffered saline, and 1 ml of 40% (wt/vol) polyethylene glycol 1540 (ATCC) in PBS-CMF was added to the pellet with gentle stirring for 1 min. After one more minute, 1 ml of Iscove's medium (without fetal calf serum) was added in 1 min with gentle stirring. Another milliliter of medium was added at the same rate. Then 8 ml of Iscove's medium (without fetal calf serum) was added at 2 ml/min. The final pellet was resuspended in selection medium and added to 96-well plates at a concentration of 2×10^5 cells per well; mouse thymocytes were used as feeders at 10^6 per well. Selection was carried out in HAT medium (44); fusions with lymphoblastoid cells were selected in HAT plus 0.5 μM ouabain.

Source of Lymphocytes. Human lymphocytes used in fusion studies included peripheral blood lymphocytes (PBL) stimulated by pokeweed mitogen (PWM), normal human spleen B lymphocytes, antigen-primed PBL and spleen lymphocytes, cells derived from normal human lymph nodes or lymph nodes involved by follicular lymphomas, and an uncloned line, C-10, of anti-tetanus toxoid antibody-producing, EBV-transformed lymphoblastoid cells.

‡ Daniels, P. J. L., Yehasbel, A. S. & Morton, J. B., Proceedings of the 13th International Conference on Antimicrobial Agents and Chemotherapy, 1973, Washington, DC, abstr. 137.

For PBL preparation, donor peripheral blood cells were collected, washed, and resuspended in RPMI 1640 medium. Ficoll/Hypaque (Pharmacia) was used as an underlayer, and the cells were centrifuged at 400 × *g* for 30 min. The cells at the interface were collected and washed. For mitogen stimulation, 1% PWM (GIBCO) was added to human PBL (10^6 cells per ml) in RPMI 1640 medium with 10% human serum and incubated at 37°C for 2–6 days before fusion.

Lymphocytes derived from lymph nodes were prepared as follows: the fatty and fibrous tissue was dissected away from the node, which was then minced with a fine scissors. The lymphocytes were released by pressing this minced tissue between two glass slides. The resulting cell suspension was then filtered through a nylon mesh (Tetko, Elmsford, NY) before fusion.

EBV Transformation. The B95-8 strain of EBV was used to transform human B lymphocytes as described (16).

Other Methods. Secretion of human IgM, IgG, or both was determined by the enzyme-linked immunosorbent assay (ELISA) procedure (45) using peroxidase-conjugated goat anti-human IgM and IgG as secondary antibodies and 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid) (Sigma) as the substrate. Newly synthesized immunoglobulins were labeled with [^{35}S]methionine or [^{14}C]leucine, and sodium dodecyl sulfate/polyacrylamide gel electrophoresis was carried out according to Laemmli (46). Gels were dried under reduced pressure and autoradiographed. Chromosome preparations were made by standard methods.

RESULTS

The heteromyelomas obtained after fusion of clone E-1 with X63-Ag8.653 mouse myeloma cells had doubling times between 15 and 22 hr. Their ouabain sensitivities varied considerably; those most insensitive were about as resistant as the parental mouse myeloma. As expected, all of the hybrids remained HAT sensitive, since selection was carried out in the absence of HAT. The heteromyelomas resulting from fusion of E-1 with unirradiated mouse myeloma cells contained variable total numbers of chromosomes, ranging between 50 and 110; only 1 human chromosome was present in most of them, but some

Table 1. Chromosomes of mouse–human myeloma cell hybrids

Cell line	Irradiation of mouse myeloma before fusion	Modal no. of chromosomes per cell	
		Mouse	Human
Parental			
FU-266 (E-1)		0	44
X63-Ag8.653-NP		57	0
Hybrid clones			
A-3, -13	−	51, 52	1
B-2, -4, -7	−	54	1
A-4, -5, -7, -8, -9; B-1	−	55	1
A-10, -11	−	56, 57	1
A-1, -14	−	57, 59	2
A-2	−	61	1–2
A-12, A-6	−	90, 98	2–3
D-6, -34	+	50, 54	1
D-24	+	51	2
D-22, -35	+	56, 57	1
D-38, -76	+	56, 59	3
D-33, -48	+	83, 84	4–5
D-36, -71	+	109, 114	7–8
D-78	+	70	10
D-3	+	124	12

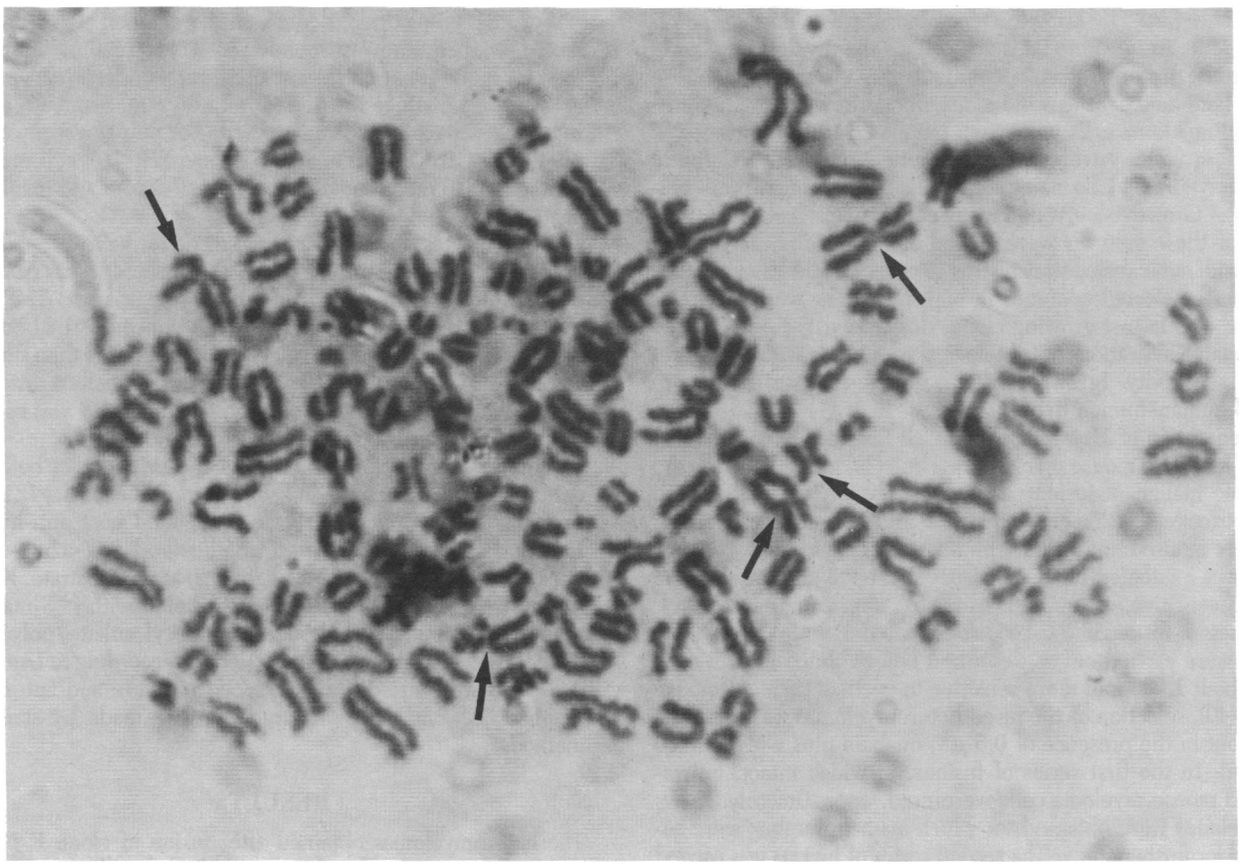

FIG. 1. Chromosome spread of heteromyeloma D-3, showing over 100 mouse chromosomes together with a few human chromosomes (arrows).

contained 2 or 3 human chromosomes (Table 1). The heteromyeloma clones obtained after fusion of irradiated X63-Ag8.653 cells with clone E-1 tended to retain a greater number (3–12) of human chromosomes (Fig. 1). The heteromyelomas are routinely grown in the presence of G-418 to promote retention of their human chromosomes. Surface membrane and cytoplasmic immunofluorescence studies, using heterologous antibodies prepared against the FU-266 human myeloma cell line, demonstrated the presence of human antigens in most of the heteromyelomas. Although all of the heteromyeloma clones initially produced human IgE(λ), some (such as D-33) became nonproducers after a few weeks in culture and others (D-36) lost ε heavy chain but retained a low level of λ light chain secretion.

Certain of the heteromyeloma clones have been consistently superior to others, and to the parental mouse myeloma cell line, in test fusions with human B lymphocytes (Table 2). Of these, clones D-33 and D-36 from the second set of heteromyeloma fusions and clone A-6 from the first set have consistently provided a high yield of viable hybrids (Fig. 2). Some heteromyelomas (A-10, B-6, D-3) have occasionally given good results but have been less consistent, and several others (D-31, D-39, D-49, D-70, D-71, D-78) have yielded few or no viable hybrids. Stability of immunoglobulin secretion and of human chromosome retention was enhanced by subcloning (Table 3). A fusion of heteromyeloma clone A-10 with PWM-PBL yielded 15 human immunoglobulin-secreting hybridomas, which were tested at serial intervals and remained stable producers for over 9 months. In another fusion involving heteromyeloma A-6 and the uncloned C-10 human lymphoblastoid cell line, hybridoma 77 produced specific IgM(κ) monoclonal antibody against tetanus toxoid for over 7 months. High percentages of immunoglobulin-secreting hybridomas were also obtained in fusions of clone D-33 with cells from lymph nodes involved by follicular lymphomas, permitting the "rescue" of their tumor-specific idiotypic immunoglobulin (5). Biosynthetic radiolabeling of such hybridomas with [^{14}C]leucine revealed monoclonal human μ and λ chains in autoradiograms of polyacrylamide electropho-

Table 2. Fusions of heteromyeloma clones with human B lymphocytes

Partner cells		Wells with viable	%	%
B Lymphocyte	Malignant	hybrids*	viable	Ig$^+$
PWM-PBL	M/H D-36	107/110	97	91
	M/H D-3	37/100	37	28
	M/H A-6	70/108	65	42
	M/H A-10	22/100	22	41
	M/H D-33	61/102	61	56
	M/H D-71	6/101	6	40
	M/H D-78	0/100	0	—
	X63-Ag8.653-NP	30/96	31	33
LCL C-10	M/H D-36	110/110	>100†	100
	M/H D-3	52/100	47	27
	M/H A-6	101/110	92	70
	M/H A-10	56/110	51	20
	M/H D-33	60/60	>100†	100
	X63-Ag8.653-NP	70/102	70	46

PWM-PBL, PWM-stimulated PBL; M/H, mouse–human heteromyeloma.
* No. wells with viable hybrids/no. wells seeded.
† All wells contained multiple clones.

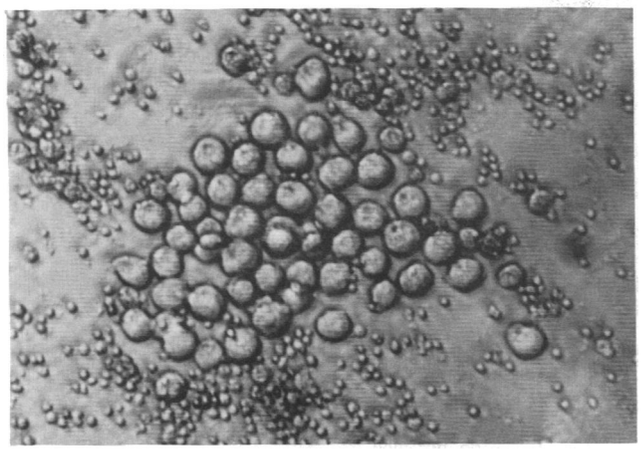

FIG. 2. Early hybridoma growing out at 7 days after fusion of heteromyeloma D-33 with lymphocytes from a lymph node involved by a follicular lymphoma. (×100.)

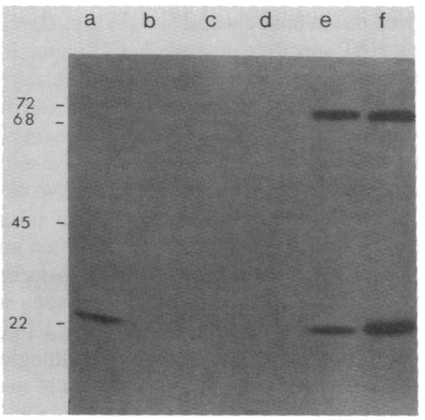

FIG. 3. Autoradiogram of sodium dodecyl sulfate/polyacrylamide electrophoresis gel of biosynthetically labeled human immunoglobulins secreted by a human myeloma line and by two hybridomas but not by a mouse myeloma line or two heteromyeloma clones. Cultures of 10^6 cells each were labeled with [^{14}C]leucine in leucine-deficient medium for 20 hr. The supernatant fluids were immunoprecipitated with 100 μl (1 mg/ml) of rabbit anti-human μ, α, ε, λ, and κ chain antibody added at 1:1 (vol/vol) ratio. Lane a, FU-266, clone E-1, IgE(λ) human myeloma. Lane b, X63-Ag8.653-NP mouse myeloma. Lane c, heteromyeloma D-36 (the concentration of λ light chain secreted by these cells is not detected by this exposure). Lane d, heteromyeloma D-33. Lane e, IgM(λ)-secreting hybridoma derived by fusion of heteromyeloma D-3 with human lymph node lymphocytes. Lane f, IgM(λ)-secreting hybridoma clone 9C10 (1F8) derived by fusion of heteromyeloma D-33 with peripheral blood lymphocytes from a patient with systemic lupus erythematosus; many of the hybridomas resulting from this fusion produced human monoclonal antibodies with specific reactivity for double- and single-stranded DNA and for ribosomal RNA. Numerals indicate marker protein molecular masses in kilodaltons.

resis gels (Fig. 3). Many hybridoma clones derived from fusions with D-33 produced between 2 and 10 μg/ml per 10^6 cells per day, and isolated clones have produced as high as 21–36 μg/ml per 10^6 cells per day. Most of the hybridoma supernatants contained no detectable mouse or hyman myeloma proteins and no evidence of permuted immunoglobulin molecules. To date, human monoclonal antibodies have been produced with specific reactivities to 2,4-dinitrophenyl, to tetanus toxoid, to double- and single-stranded DNA, to ribosomal RNA, to *Escherichia coli* O111:B4(J5) endotoxin, and to Rh factor (unpublished data).

DISCUSSION

There are many important clinical applications for which human monoclonal antibodies would be highly desirable (47). However, the few human myeloma cell lines in existence (reviewed in ref. 30) appear to be clearly less than satisfactory as malignant fusion partners. In contrast, several mutant mouse myeloma cell lines are now available that are remarkably efficient and reliable as malignant fusion partners in mouse–mouse hybridoma production. Unfortunately, when these mouse myeloma cell lines are fused with antigen-primed human B lymphocytes, the capacity of the resultant mouse–human hybridomas to secrete human monoclonal antibody is often transient due to the selective elimination of human chromosomes (10, 11).

Accordingly, we have devised a strategy aimed at retaining the outstanding fusion characteristics of the HAT-sensitive mouse myeloma cell line X63-Ag8.653 (39) under selection conditions in which it is forced to retain one or more human chromosomes bearing a bacterial gene for neomycin resistance introduced by the protoplast fusion procedure. It was hoped that, in at least some of the heteromyelomas thus constructed, there would be a significant increase in the retention of human chromosomes introduced during secondary fusions with normal human B lymphocytes, thus providing a higher yield of viable mouse–human hybridomas capable of stable human immunoglobulin secretion. This expectation has now been confirmed; some heteromyelomas have consistently generated high yields of viable human immunoglobulin-secreting hybridomas, most of which grow well and can readily be subcloned. Of course, the yield of viable hybrids is also strongly dependent on the efficiency with which the normal human B lymphocyte fusion partners are primed with antigens or stimulated with mitogens.

The heteromyeloma approach has had three further advantages. The hybrid progeny, like their parental mouse and human myeloma cell lines, remained HAT sensitive, since selec-

Table 3. Stability of human immunoglobulin secretion and chromosome pattern

	1–2 months			3–4 months			5–6 months			7–10 months		
Hybrid clone	γ	λ	M/H	γ	λ	M/H	γ	λ	M/H	γ	λ	M/H
A/E6	+	+	86/16	+	+	75/13	+	+	76/16	−	+	65/12
A/E6/A7				+	+	72/12	+	+	73/12	+	+	75/12
A/E6/D7				+	+	66/11	+	+	75/16	+	+	71/16
B/D1	+	+	77/12	+	+	75/10	+	+	72/11	−	+	61/12
B/D1/F10				+	+	73/14	+	+	NT	+	+	72/15
B/D1/H3				+	+	72/12	+	+	NT	+	+	64/13
B/D1/F6				+/−	+/−	72/13	−	−	65/8		NT	

Times refer to months after fusion. γ and λ indicate secretion of human IgG(λ) heavy and light chain; M/H indicates modal no. of mouse/human chromosomes; NT, not tested. A/E6/A7 and A/E6/D7 are subclones of hybridoma A/E6; subclones of hybridoma B/D1 are labeled similarly.

tion was carried out in ouabain and G-418; thus the tedious step of reselecting HAT-sensitive mutants in 6-thioguanine or 8-azaguanine was obviated. Second, the acquisition by these heteromyelomas of ouabain resistance from the murine parental myeloma permitted the hybrid progeny of fusions with EBV-immortalized human lymphoblastoid cells to be selected readily in HAT plus ouabain. Thus, antigen-reactive cells in populations of human B lymphocytes primed with antigen *in vivo* or *in vitro* can be selected by "panning" and then amplified to the numbers needed for fusion by EBV transformation (unpublished data). Finally, the heteromyelomas soon stopped secreting the ε heavy chain and usually also the λ light chain of the human myeloma parent. Thus, the immunoglobulins secreted by hybridomas resulting from the fusion of nonproducer heteromyeloma clones with antigen-primed human B lymphocytes are not diluted by the presence of human or mouse myeloma proteins.

The high yield of viable hybridomas, their capacity for sustained secretion of relatively high levels of human immunoglobulin, and the absence of permuted immunoglobulin molecules in the culture supernatants indicate that these heteromyeloma clones provide significant advantages as malignant fusion partners for human monoclonal antibody production. The heteromyeloma clone that consistently scores highest with respect to hybridoma yield, ease of cloning, and stability of immunoglobulin secretion will soon be made available to interested scientists for investigative purposes.

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