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The Use of Lymphocytes From Axillary Lymph Nodes of Mastectomy Patients to Generate Human Monoclonal Antibodies*

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Abstract—*Lymphocytes from axillary lymph nodes of breast cancer mastectomy patients were fused with murine non-immunoglobulin-secreting myeloma cells to generate hybridoma cell lines that synthesize human immunoglobulins. Lymph node lymphocytes from* 21 *patients were used to obtain* 505 *human–mouse hybrid cultures. From these,* 62 *cultures were established which synthesized immunoglobulins reactive in radioimmunoassays specific for either human IgG or human IgM. Some of these double-cloned hybrid cell lines produced human monoclonal antibodies for at least* 6 months. *Sodium dodecylsulfate polyacrylamide gel electrophoresis and immunodiffusion analysis demonstrated that the monoclonal antibodies possessed human heavy and light immunoglobulin chains. Levels of synthesis ranged from* 0.1 *to* 20 μg *of human immunoglobulin per* ml *of culture fluid. The immunoreactivity of some of these human monoclonal antibodies with mammary carcinoma cells is summarized and has been documented elsewhere* (J. SCHLOM, D. WUNDERLICH and Y. A. TERAMOTO. *Proc. Natl Acad Sci USA* 1980; **77:** 6841); *the reactivity of the majority of the immunoglobulins, however, is still unknown at this time. The studies reported here detail the procedures in which axillary lymph nodes from mastectomy patients are used in the generation of human–mouse hybridomas that synthesize human monoclonal antibodies.*

INTRODUCTION

THE TECHNIQUE of monoclonal antibody production via cell fusion, introduced by Kohler and Milstein [1, 2], has revolutionized approaches to the serologic analysis of complex antigens and the immunodiagnosis and immunotherapy of disease. It is now theoretically possible to produce specific monoclonal antibodies against an unlimited number of antigens and antigenic determinants. One limitation, however, has been the lack of a source of human lymphocytes needed for the production of antigen-specific human monoclonal antibodies. Past reports have demonstrated that hybrid cell lines generated by the fusion of human peripheral blood lymphocytes or human leukemic lymphocytes with immunoglobulin-secreting mouse myeloma cells did secrete human immunoglobulin components. These cell lines, however, also secreted mouse immunoglobulin components and mixed mouse–human immunoglobulin molecules [3, 4].

In this report we detail the fusion of human lymphocytes, obtained from axillary lymph nodes of breast cancer mastectomy patients, with a non-immunoglobulin-secreting murine myeloma cell line; these fusions give rise to human–mouse hybridomas that synthesize and secrete complete human immunoglobulin molecules. After double cloning, the hybridomas remain stable producers of human (and not murine) monoclonal antibodies for at least six months.

MATERIALS AND METHODS

Reagents

RPMI-1640 medium supplemented with 15% fetal calf serum, 100 units per ml penic-

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illin, 100μg per ml streptomycin, 0.25 μg per ml fungizone and 1mM sodium pyruvate (designated Complete RPMI) was used for culturing all cells. HAT media consisted of Complete RPMI supplemented with 1×10^{-4} M hypoxathine, 4×10^{-7} M aminopterin and 1.6×10^{-5} M thymidine. HT medium consisted of Complete RPMI supplemented with 1×10^{-4} M hypoxanthine and 1.6×10^{-5} M thymidine. Polyethylene glycol-1500 was purchased from BDH Chemical Ltd., Poole, U.K. Rabbit anti-human IgM (mu chain specific) and rabbit anti-human IgG (gamma chain specific) immunobeads were purchased from Bio-Rad Laboratories, Richmond, CA. Chromatographically purified human IgM (pentamer), and human IgG, purchased from Cappel Laboratories, Cochranville, PA, were labeled with ^{125}I-Na by the iodogen technique [5] and were used to generate standard curves in the immunobead competition radioimmunoassay. Rabbit anti-human IgM (mu chain specific), rabbit anti-human IgG (gamma chain specific), $F(ab)'_2$ fragments of goat anti-human Fab, rabbit anti-human kappa light chains and rabbit anti-human lambda light chains were also purchased from Cappel Labs. Mouse IgG and IgM were purchased from Litton Bionetics, Kensington, MD. Protein A from *Staphylococcus aureus* was purchased from Pharmacia, Piscataway, NJ, and was iodinated using the iodogen method.

Cells and hybridoma methods

Segments of human axillary lymph nodes (approximately one quarter of each of four to eight nodes) were obtained aseptically from patients undergoing modified radical mastectomy. Excess fat was trimmed from the nodes and a single cell suspension was prepared by straining the tissue through a No. 3 stainless steel mesh. The BALB/c non-immunoglobulin-secreting cell line NS-1 [6] was kindly supplied by Dr. J. Kim, National Institutes of Health, Bethesda, MD. Human lymph node cells and NS-1 cells, in log phase of growth, were mixed at a 4:1 ratio and fused with polyethylene glycol-1500 as previously described [7]. After fusion, the equivalent of 10^6 total cells (as determined by viable cell counts prior to fusion) were seeded into individual wells of 96 well microtiter plates (Costar Plastics, Cambridge, MA) in 100 μl of Complete RPMI. Twenty-four hours after fusion, 100 μl of HAT media was added to each well. On days 2, 3, 5, 8, 11, 14, 17 and 21 post fusion, 100 μl of media was removed from each well and replaced with 100 μl fresh HAT media. Hybrid cells usually appeared between days 10 and 14. Supernatant fluids were subsequently assayed for the presence of human immunoglobulin.

Cultures positive for human immunoglobulin production were transferred to wells 16 mm in diameter (Costar Plastics, Cambridge, MA) in HT media. C57BL/6 mouse thymocytes (1×10^6 per well) were used as feeder cells. Thereafter, hybrid cells were transferred to 25 cm^2 and 75 cm^2 culture flasks and aliquots were viably frozen in Complete RPMI containing 7.5% dimethylsulfoxide (DMSO) at various passage levels.

Human–mouse hybridomas were cloned by limiting dilution. A minimum of four 96 well plates were seeded at approximately 0.5–1 cells per well. C57BL/6 thymocytes (1×10^6 per well) were used as feeder cells. Seven to ten days after cloning, plates were examined for the presence of hybridoma colonies. Employing this method, approximately one well in two contained subsequent hybridoma colonies. The supernatants from wells containing one colony were harvested and assayed for the presence of human immunoglobulin. Hybridoma cultures were cloned a minimum of two times.

Immunobead competition radioimmunoassays

Supernate fluids from hybridoma cultures were assayed for the presence of human immunoglobulin by their ability to compete for the binding of ^{125}I-labeled human immunoglobulin (IgG or IgM, respectively) to rabbit anti-human immunoglobulin (IgG or IgM, for specific assays, respectively) covalently bound to polyacrylamide microspheres (immunobeads). The immunobead suspensions were used at the dilution which bound 20% of a 15,000 cpm input of ^{125}I-labeled human immunoglobulin.

One hundred microliters of hybridoma supernate fluids was diluted to 500 μl with standard diluent [TNE (0.01 M Tris, pH 7.2, 0.1 M NaCl, 0.001 M EDTA) containing 0.1% Triton X-100 and 0.2% BSA]. Subsequent dilutions were in a mixture of Complete RPMI and standard diluent at a 1:5 ratio. One hundred microliters of the hybridoma supernate dilutions and 50 μl of the immunobeads, suspended in phosphate-buffered saline, pH 7.2 (PBS), were incubated for 2 hr at 37°C in 0.4 ml microtiter analyses test tubes (Walter Sarstedt Inc., Princeton, NJ). Fifteen thousand cpm of ^{125}I-human immunoglobulin (IgG or IgM, each to its respective immunobead assay) in 5 μl were

then added. After incubation for an additional 2hr at 37°C, the reaction mixtures were centrifuged at 10,000*g* for 1.5 min. Supernates were aspirated and the amount of ^{125}I-labeled human immunoglobulin bound to the beads (in the pellet) was determined by assaying the tubes for radioactivity in a Beckman auto gamma counter. All samples were assayed in duplicate at several dilutions. Duplicates were averaged, backgrounds were subtracted, and data normalized so that maximum binding (as determined by employing a 1:5 dilution of Complete RPMI in standard dilutent as competitor) was defined as 100%.

Solid phase radioimmunoassays

Hybridoma supernatant fluids were also assayed for human IgM or human IgG by solid phase binding assay employing ^{125}I-labeled Protein A (IPA). Fifty microliters of F (ab)'$_2$ fragments of goat anti-human Fab (at 8.75 μg/ml in PBS) was dried onto individual wells of 96-well soft plastic microtiter plates (Dynatech Laboratories, Alexandria, VA) by incubation for 16 hr at 37°C. Wells were then coated with 100 μl of 5% BSA in PBS; this reduced nonspecific protein absorbtion during the course of the assay. Fifty microliters of hybridoma supernatant fluid was then added to the coated wells in triplicate and incubated for 1hr at 37°C. After washing with 1% BSA in PBS, 50 μl of a 1:15,000 dilution of rabbit anti-human IgG in PBS was added to one well, 50 μl of a 1:5000 dilution of rabbit anti-human IgM in PBS was added to the second well, and 50 μl of PBS was added to the third well. After incubation for 1hr at 37°C, wells were washed four times with 1% BSA and 50,000 cpm of IPA in 25 μl was added to each well. After incubation for an additional hour at 37°C, the unbound IPA was removed from the wells by washing three times with 1% BSA in PBS. Bound IPA was detected in two ways: (a) autoradiography of the plates for 16 hr at −70°C on Kodak XR-5 film with enhancement by Dupont Cronex intensifying screens, or (b) cutting individual wells from the plate and assaying for radioactivity in a Beckman auto gamma counter.

Velocity sedimentation analysis of human monoclonal antibody

One hundred microliters of hybridoma supernatant fluids was centrifuged for 16 hr through a 5 ml 10–40% sucrose gradient. Individual 0.2 ml fractions were collected from the gradient by bottom puncture and tested in solid phase radioimmunoassay for human immunoglobulin content. Wells were cut from the plates and assayed for amount of radioactivity bound. Polyclonal human IgM and IgG were run on parallel gradients as markers.

SDS–PAGE of human monoclonal antibody

Human monoclonal antibodies were internally labeled by incubating hybridoma cultures in amino acid free RPMI-1640 medium containing 1% dialyzed fetal calf serum and 40 μCi per ml of mixed ^{3}H-amino acids (New England Nuclear Corp., Boston, MA) for 16 hr at 37°C. Supernates were collected and incubated with immunobeads (specific for that class of antibody being labeled) for 2 hr at 37°C. The beads were pelleted and antibody eluted by incubation in 1% SDS for 3 hr at 37°C. The beads were then removed by centrifugation and supernatants subjected to SDS–PAGE as described elsewhere [8]. After electrophoresis, gels were cut into 1 mm slices, incubated overnight at 37°C in 3% (vol/vol) protosol (New England Nuclear Corp.) in formula-949 scintillation cocktail (New England Nuclear Corp.), and assayed for radioactivity in a Beckman LS-233 scintillation counter.

Immunoperoxidase procedure

Five-micrometer sections of formalin-fixed tissues on slides were deparaffinized and incubated with 3% H_2O_2 in methanol to block endogenous peroxidase activity [9]. After rinsing in PBS, the slides were incubated with a 1:5 dilution of normal goat serum (NGS) for 15 min. The NGS was removed and human monoclonal antibody (0.5–1.6 μg in 0.1 ml) or polyclonal human IgM (used at an identical Ig concentration as a negative control) was added for 30 min. The slides were then rinsed in PBS and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-human IgG (gamma chain specific) or anti-human IgM (mu-chain specific) for 30 min. The sections were then rinsed in PBS, reacted with 0.06% diaminobenzidine and 0.01% H_2O_2 for 5 min and rinsed in PBS; they were then counterstained with hematoxylin, dehydrated and mounted.

RESULTS

Generation and propagation of human–mouse hybridomas

Lymph nodes were obtained aseptically from 21 patients within 1–20 hr of modified

Table 1. Pathology reports

Patient	Age	Diagnosis	Positive nodes*
MB	41	Infiltrating poorly differentiated ductal carcinoma	13/17
RP	63	Infiltrating ductal carcinoma/fibroadenoma	4/9
SG	40	Infiltrating duct carcinoma/fibrocystic disease with foci of intraductal hyperplasia/sinus histiocytosis	0/10
EW	55	Mixed infiltrating ductal and lobular carcinoma/ extensive lymphatic involvement by carcinoma	3/14
GR	73	Infiltrating ductal carcinoma	9/9
TC	46	Mixed infiltrating lobular and ductal carcinoma	2/9
TW	44	Infiltrating ductal carcinoma	0/13
MH	67	Infiltrating ductal carcinoma	0/8
TM	70	Infiltrating ductal carcinoma	1/9
KC	42	Poorly differentiated invasive duct adenocarcinoma/ one intraductal papilloma bearing focal squamous metaplasia/fibrous mastopathy	0/10
EP	67	*In situ* and infiltrating ductal carcinoma, comedo type/ fibrocystic mastopathy	0/4
LG	54	Infiltrating ductal carcinoma	0/17
MY	55	Malignant sclerosing ductal cell carcinoma/ fibrocystic disease	0/17
WS	58	Infiltrating carcinoma, suggestive of lobular carcinoma	18/18
VF	58	Infiltrating ductal carcinoma	0/32
EH	57	*In situ* lobular and terminal duct carcinoma	0/14
RE	39	Infiltrating lobular carcinoma	18/19
MW	61	Papillary carcinoma	0/21
CF	66	Intraductal adenocarcinoma	4/20
MS	67	Infiltrating lobular carcinoma	0/12
WI	82	Infiltrating carcinoma	9/9

*No. of lymph nodes positive for metastases per total No. nodes examined.

radical mastectomy. Table 1 lists the ages, pathological diagnosis and metastatic nodal involvement of the patients from whom specimens were obtained. Fusion of the human lymphocytes and murine NS-1 cells was performed as described in Materials and Methods. Hybridomas usually appeared 10–14 days after fusion as colonies of refractile cells hidden beneath cellular debris. In all, 2060 wells were seeded from the fusion products of 21 patients (Table 2). Of these, 505 wells (24%) yielded hybrid cells that could be propagated. In a few of the fusions, hybrid cells never appeared in any of the wells seeded (patients GR, TW and MH; Table 2). In the successful fusions, the number of wells where hybrid colonies were detected ranged from less than 1% to 55% of the wells seeded. The reasons behind this variability in fusion efficiency are not known at this time.

Clumping of the hybrid cells was observed in a vast majority of wells that contained hybrid colonies. The contents of these wells were routinely resuspended and transferred to an additional microtiter well. Transferring the

Table 2. Generation of human–mouse hybrids

Patient	No. wells seeded	No. wells with hybridomas*
MB	100	40 (40)
RP	120	29 (24)
SG	120	3 (<1)
EW	110	3 (<1)
GR	60	0 (0)
TC	150	30 (20)
TW	100	0 (0)
MH	90	0 (0)
TM	90	26 (29)
KC	90	19 (21)
EP	100	31 (31)
LG	100	25 (25)
MY	60	25 (42)
WS	60	26 (43)
VF	60	33 (55)
EH	280	128 (46)
RE	77	8 (10)
MW	103	31 (30)
CF	80	8 (10)
MS	70	33 (47)
WI	40	7 (18)
Total 21	2060	505 (24)

*Percentage of wells with propogating hybridomas in parentheses.

hybrids in this manner reduced cellular debris, disaggregated clumps, and exposed hybrid cells that were previously hidden beneath the dead cells and debris.

Production of human immunoglobulin by hybridomas

Two screening methods were used to determine if human IgG or IgM were being secreted into culture supernatant fluids of the hybrid cell lines: an immunobead competition radioimmunoassay, and a solid phase binding assay. Figures 1(A) and (B) demonstrate the relative specificities of the immunobead competition radioimmunoassays for human IgG and human IgM, respectively. The immunobead assay for human IgG could detect as little as 10 ng of human IgG per ml (Fig. 1A). Mouse IgG did not compete in the assay. Human IgM did compete in the IgG assay to some extent; however, this is most likely due to IgG contamination of the IgM preparation (obtained from sera of myeloma patients). The IgM immunobead assay could detect as little as 25 ng human IgM per ml (Fig. 1B). Human IgG did not compete in the assay and mouse IgM competed only slightly at an input of 1000 ng/ml: Fig 2 shows competition by representative hybridoma supernatant fluids in the human IgG and IgM immunobead assays. Immunoglobulins from supernatant fluids of cultures C and D compete completely in the human IgG assay but are negative in the human IgM assay. The reciprocal is true for immunoglobulins obtained from supernatant fluids of hybridoma cultures A and B (Fig. 2).

Further evidence for the synthesis and secretion of human immunoglobulins by human–mouse hybridoma cultures was obtained using the solid phase binding assays described in Materials and Methods. As shown in Fig. 3, the immunoglobulins from hybridoma cultures A and B are human IgM's, since Protein A binds only when rabbit anti-human IgM is used as a second reagent. In the case of culture fluid C, reactions of similar intensity are observed when rabbit anti-human IgM and PBS are used as second reagents; the reaction intensity is greatest, however, when the second reagent used is rabbit anti-human IgG. Since protein A has been shown to bind to human IgG subclasses 1, 2 and 4 [10], IgG_1, IgG_2 or IgG_4 will elicit a positive reaction regardless of the second reagent used in the solid phase radioimmunoassay. This is the case with culture fluid C (Fig. 3); the intensity of the reaction with anti-human IgM and PBS as the second reagent merely reflects the binding of Protein A to the human IgG_1, IgG_2 or IgG_4 monoclonal antibody. Protein A, on the other hand, does not react with human IgG subclass 3 [9]; immunoglobulin from culture supernatant D appears to be of this subclass; binding of Protein A is observed only when rabbit anti-human IgG is used as a second reagent (Fig. 3). Figure 3 also demonstrates the specificity of the solid phase radioimmunoassay. Mouse IgG and mouse IgM do not react in the solid phase assay.

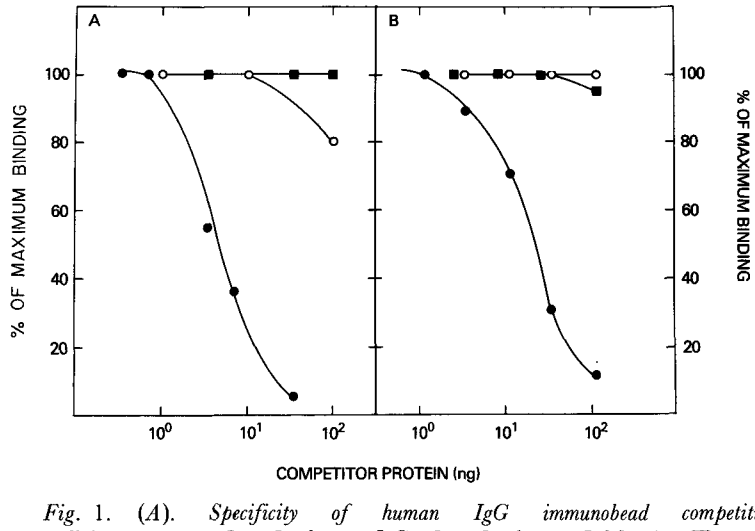

Fig. 1. (*A*). *Specificity of human IgG immunobead competition radioimmunoassay:* ●—●, *human IgG;* ○—○, *human IgM;* ■—■, *mouse IgG.* (*B*) *Specificity of human IgM immunobead competition radioimmunoassay:* ●—●, *human IgM;* ○—○, *human IgG;* ■—■, *mouse IgM.*

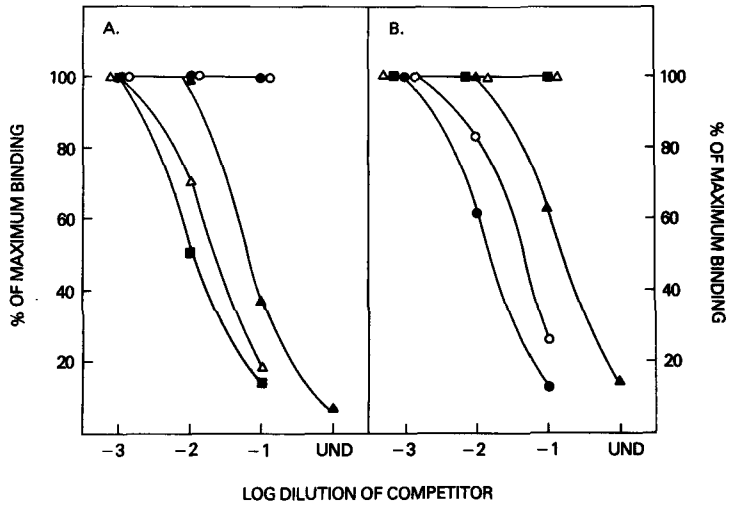

Fig. 2. Competition by human monoclonal antibodies in immunobead assays. (A) Human IgG immunobead competition radioimmunoassay: ▲—▲, human IgG, undiluted = 100 ng; Supernatant fluids from: ●—●, hybridoma culture A; ○—○, hybridoma culture B; △—△, hybridoma culture C; ■—■, hybridoma culture D. (B) Human IgM immunobead competition radioimmunoassay: ▲—▲, human IgM, undiluted = 100 ng; ●—●, hybridoma culture A; ○—○, hybridoma culture B, △—△, hybridoma culture C; ■—■, hybridoma culture D.

Approximately 12% (62/505) of the hybridoma culture supernatant fluids tested were positive for human IgG or human IgM (Table 3). None of the hybrid cultures tested produced a mixture of immunoglobulins. The hybrid cell cultures derived from lymphocytes from a few patients, on the other hand, synthesized neither IgM nor IgG. More cultures were established that synthesized human IgM than those synthesizing human IgG. Furthermore, cultures established from lymph nodes of some patients synthesized predominantly human IgM, while cultures from other patients synthesized predominantly IgG (Table 3). The reasons for this are unknown at this time.

As expected, production of human immunoglobulin was not a stable property of most human–mouse hybridomas. Figure 4 shows the duration of immunoglobulin production by 52 cultures obtained from the fusion products of 16 patients. Twelve of the human hybridoma cell lines ceased synthesizing human immunoglobulin between days 1 and 20, and 17 more stopped production before day 60. Twenty-three cell lines, however, did continue to secrete human Ig's through at least the 61 day observation period. The degree of stability of various clones for immunoglobulin production is exemplified in Table 4. As can be seen, out of twenty-one primary clones that were producing human IgM on day 71, only seven remained positive on day 94. Upon recloning, however, 126 out of 129 secondary clones continued to secrete human IgM through day 220.

Levels of immunoglobulin synthesis varied among individual human–mouse hybridomas. Approximately one-half of the cultures synthesized between 0.1 and 1.0 μg of human

Table 3. Production of human immunoglobulins by human–mouse hybridomas

Patient	No. cultures tested	No. cultures positive for: Human IgM	Human IgG
MB	40	5	1
RP	29	2	1
SG	3	1	0
EW	3	0	0
TC	30	3	1
TM	26	7	6
KC	19	1	7
EP	31	2	1
LG	25	2	4
MY	25	2	0
WS	26	2	1
VF	33	3	0
EH	128	5	2
RE	8	0	0
MW	31	1	0
CF	8	0	0
MS	33	0	0
WI	7	1	1
Total 18	505	37	25

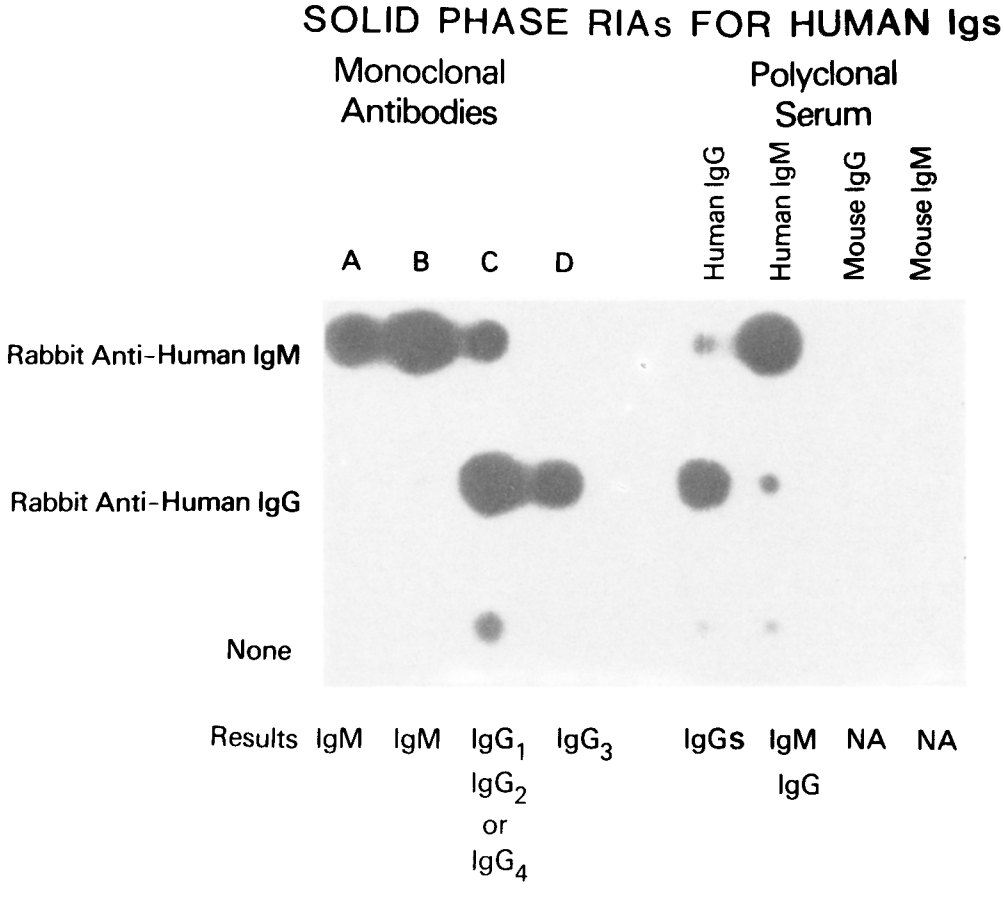

Fig. 3. Solid phase radioimmunoassay for human IgG and human IgM. See Materials and Methods for details. Polyclonal serum samples tested at inputs of 50 ng. *NA = Not applicable.*

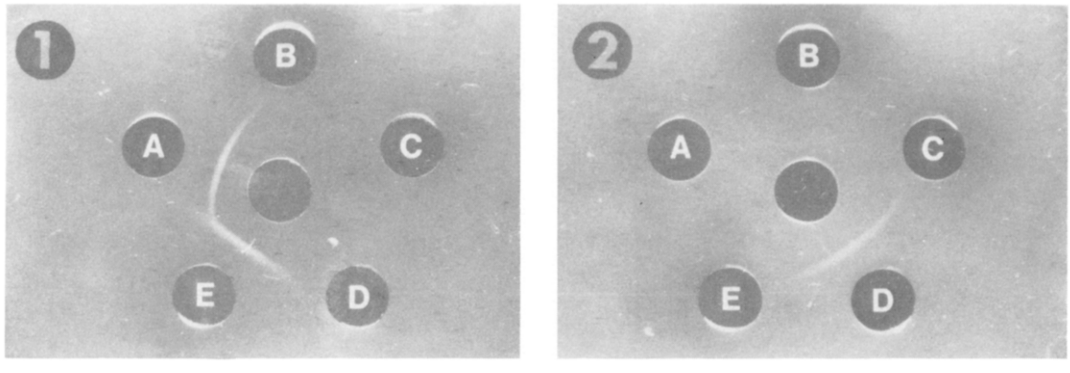

Fig. 7. Ouchterlony analysis of human IgM monoclonal antibody. Panel 1: *Center well:* 40 µl *of* 10× *concentrate of hybridoma culture fluid. Panel* 2: *Center well*: 40 µl *of purified murine IgM. The contents of the outer wells for panels* 1 *and* 2 *are:* (*A*) 20 µl *of rabbit anti-human mu chain;* (*B*) 20 µl *of rabbit anti-human gamma chain;* (*C*) 20 µl *of rabbit anti-human kappa chain;* (*D*) 20 µl *of rabbit anti-mouse IgM; and* (*E*) 20 µl *of rabbit anti-human lambda chain.*

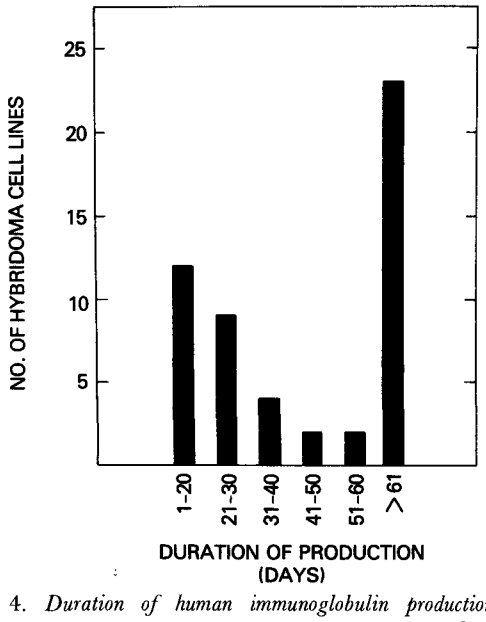

Fig. 4. Duration of human immunoglobulin production by hybridoma cell lines. Fifty-two hybridoma cell lines from 16 patients were propagated and assayed twice per week for the presence of human IgG or human IgM.

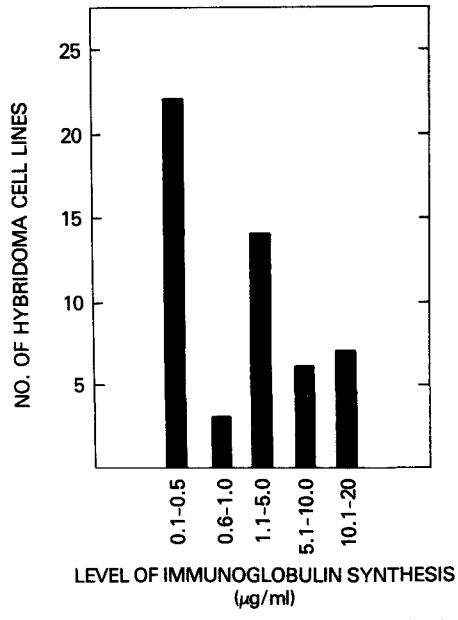

Fig. 5. Level of human immunoglobulin synthesis by human–mouse hybridoma cell lines. Culture fluids from 52 hybridoma cell lines tested for human IgG or IgM content using the immunobead competition radioimmunoassay.

immunoglobulin per ml (Fig. 5). The vast majority of these were also the cell lines that ceased immunoglobulin production the earliest. Twenty cultures (38%), however, synthesized between 1.1 and 10 μg human immunoglobulin per ml and seven others (14%) produced human immunoglobulins at levels between 10.1 and 20 μg/ml of culture fluid.

Human hybridoma cell lines were easily adapted to mass culture, i.e., growth in roller bottles. Experiments were also undertaken to determine if the cell lines could be cultured in serum-free media in mass culture. The absence of serum proteins would simplify the purification of the monoclonal antibodies from the culture medium. Cell lines were grown to confluence in serum-containing medium and then transferred to two additional culture vessels containing serum-free medium. Cells were then cultured in the serum-free medium

Table 4. Propagation and cloning of a human–mouse hybridoma cell line

Day	Operation	Result
0	Fusion of human lymphocytes and NS-1	100 microtiter wells planted
14	Wells visually examined for growth	40 wells with hybridomas
31	Assay of wells for human Ig production	6 of 40 positive for Ig production (5 for IgM, 1 for IgG)
33–40	Passage of 6 Ig positive cultures to 25 cm^2 and 75 cm^2 flasks	
57	Clone IgM producing hybrid; 960 wells planted at approx. 1 cell/well	169/960 well displayed one colony on day 65
71	169 clones assayed for IgM production	21/169 positive
72–94	Propagated clones; 16 mm wells, 25 cm^2 75 cm^2 vessels	
94	Assay of 21 cultures for IgM synthesis	7/21 positive for IgM
96	Secondary cloning of 3 highest titer IgM positive primary clones. 384 wells planted for each of the primary 3 clones at approx. 1 cell/well	73/384, 52/384 and 90/384 wells displayed one colony on day 101, respectively
106	Assay secondary clones for human IgM production	26/73, 35/52, 68/90 clones, respectively, positive for IgM synthesis
220	Assay secondary clones for human Ig synthesis	126/129 secondary clones positive for IgM production

for 48hr. Titers of human monoclonal antibodies secreted into the serum-free culture medium were approximately one-half of those obtained with comparable cultures grown in medium containing serum.

Immunoperoxidase studies

Each human monoclonal antibody was tested via the immunoperoxidase technique (see Materials and Methods) for its reactivity with human breast tumor cells and normal human tissues. The results employing one of these monoclonals (MBE6) have recently been detailed elsewhere [11]. Whereas the majority of primary (54/67) and metastatic (20/20) mammary tumor tissue sections demonstrated substantial reactivity with monoclonal MBE6, tissue sections from only 3 of 17 benign breast lesions (fibroadenomas and fibrocytic disease) demonstrated reactivity. Moreover, lactating mammary gland tissue, and apparently normal tissues from numerous sites, including normal lymph node, colon, lung, thyroid, arteries, veins and stroma, were all negative.

Analyses of human immunoglobulins

Experiments were undertaken to determine if the secretion products of the human–mouse hybridoma cultures consisted of complete human immunoglobulin molecules. Tritiated amino acids were added to hybridoma cultures and the supernatent fluids were immunoprecipitated and subjected to SDS–gel electrophoresis as described in Materials and Methods. The migration pattern of one such IgM monoclonal antibody is presented in Fig. 6(B). Peaks were obtained corresponding to molecular weights of 75,000 and 22,000, which agree with the molecular weights expected for IgM heavy and light chains, respectively. This human monoclonal antibody was also subjected to velocity sedimentation analysis to determine if the IgM synthesized was in the monomeric or pentameric form. Polyclonal human IgM (pentameric) and IgG were used as markers. As shown in Fig. 6(A), the human monoclonal antibody demonstrated a sedimentation coefficient of approximately 19S, corresponding to pentameric IgM.

Supernatant fluids fron hybridoma culture lines were further characterized via Ouchterlony double diffusion analyses. Figure 7 shows a typical reaction pattern. The human IgM monoclonal antibody (the same antibody tested in Fig. 6) reacts only with rabbit anti-human mu chain and rabbit anti-human lambda chain (Fig. 7–1). No reaction

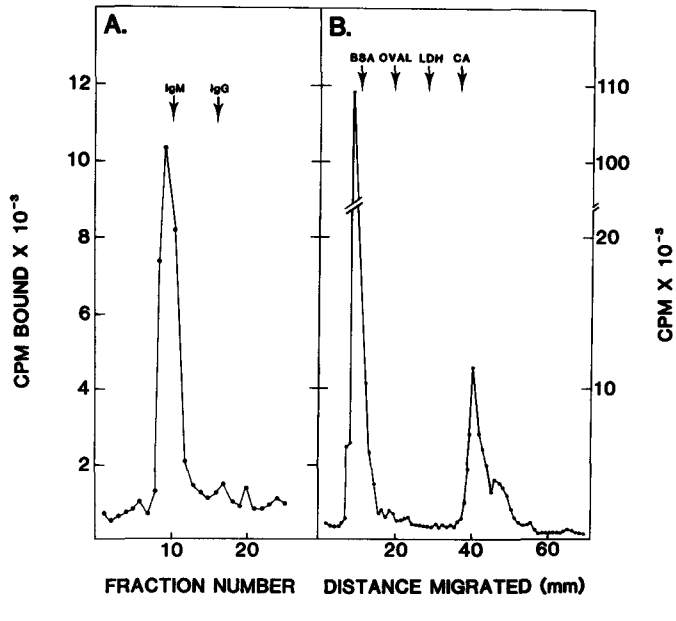

Fig. 6. SDS–PAGE and velocity sedimentation analysis of human monoclonal IgM. (A) Human monoclonal antibody was labeled with ^{3}H-*amino acids, immunoprecipitated and subjected to SDS–PAGE as described in Materials and Methods. Migration is from left to right. (B) Human monoclonal antibody was centrifuged through* 10–40% *sucrose gradient and fractions tested for human IgM content. Pentametric human IgM and human IgG were used as markers.*

was observed against rabbit anti-human gamma or kappa chains or rabbit anti-murine IgM. Figure 7–2 demonstrates the specificity of the reagents that were used. Purified murine IgM reacts only with rabbit anti-murine IgM, and not the anti-human reagents. Thus, no evidence of murine immunoglobulin is detected in the human monoclonal antibody preparation.

DISCUSSION

In this study we have demonstrated that human lymphocytes, obtained from lymph nodes of modified radical mastectomy patients, can be employed in the generation of stable human–mouse hybridoma cell lines that secrete human monoclonal antibodies for at least six months. Fusions of the human lymphocytes with the mouse non-producer myeloma cell line were carried out using techniques and reagents similar to those employed in our laboratory for generating mouse–mouse and mouse–rat hybridomas. The hybrid cells did, however, require constant monitoring during the 96–well stage immediately after the fusion. The hybrid cells tended to clump and form large clusters of cells. Routine resuspension and transfer of the wells containing hybrid cells appeared to ensure their survival. The number of fusion wells yielding hybrid cells varied for individual patients from zero to over 50% of the wells planted. There is no outstanding correlation observed at this time between pathological description of the tumor mass, age of patient or metastatic nodal involvement, and the success of the fusion; our sampling was small, however, and larger numbers of samples may be needed to define such correlations if they exist. Sixty-two of the hybridoma cultures established synthesized and secreted human immunoglobulins: 7.3% of the hybrid cultures produced human IgM and 4.7% produced human IgG. It has been reported that of the B lymphocytes present in axillary nodes containing breast tumor metastases, the IgM-bearing lymphocytes are predominant [12, 13]. This could be one of the factors that accounted for the predominance of IgM-secreting hybridomas that we observed.

Cloning of the human hybridomas was done by limiting dilution. An important practice here appeared to be the planting of numerous cloning wells; simply, the more wells planted, the greater the chance of obtaining a stable immunoglobulin-producing cell line. In one hybrid cell line, over 65% of primary clones ceased producing human immunoglobulin over a period of three weeks. After two clonings, however, we were able to establish human–mouse hybridoma cell lines that produced human immunoglobulins for at least six months.

A previous report [14], involving the production of rabbit–mouse hybridomas, has suggested that hybridomas obtained from interspecies fusions may not synthesize complete immunoglobulin molecules. In order to investigate the properties of the immunoglobulins produced by the human–mouse hybridomas, SDS–PAGE, velocity sedimentation analysis and Ouchterlony analysis was performed. SDS–PAGE, after internal radioactive labeling of immunoglobulins, demonstrated that one IgM-producing cell line synthesized both heavy and light chains. Ouchterlony double diffusion demonstrated that these heavy and light chains reacted with anti-human reagents, and sedimentation analysis showed that the IgM molecules being synthesized were in the pentameric form. Not all hybridoma cell lines have been tested in this manner; studies are currently underway to determine the number of cell lines that are producing intact human immunoglobulins.

Based on the hypothesis that the lymphocytes from lymph nodes draining primary breast tumors may be primed against breast tumor cell antigens, it is possible that some of the human monoclonal antibodies described here may be reactive against human breast tumor cells. At least one human IgM monoclonal antibody did bind preferentially to breast tumor cells from the patient from which the nodes were obtained. In addition, this antibody was also reactive with carcinoma cells from other breast tumors. These studies have been described in detail elsewhere [11]. The studies reported here do demonstrate, however, that stable hybridoma clones synthesizing considerable levels of complete human monoclonal antibodies can be derived from human–mouse fusions employing lymphocytes obtained from lymph nodes of mastectomy patients.

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