

# Production of Autoantibodies by Human-Human Hybridomas

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**ABSTRACT** Peripheral blood lymphocytes and splenocytes of patients with autoimmune disease were used to prepare human-human hybridomas that produce autoantibodies. Because exogenous immunization was not used, the hybridoma antibodies were derived from B cells that spontaneously produced autoantibodies. 108 hybrids grew from 4,254 wells (2.5%). Optimal conditions for obtaining hybridomas with the GM 4672 myeloma line included initial growth in 2-ml wells, the use of 44% polyethylene glycol, a mononuclear cell/GM 4672 cell ratio of 5:1, and prior stimulation of the B lymphocytes with pokeweed mitogen. Hybridoma supernatants had activity against ssDNA, platelets, and erythrocytes. The results demonstrate the feasibility of producing human-human hybridomas from lymphocytes of patients with various autoimmune diseases.

## INTRODUCTION

Conventional applications of hybridoma technology require the stimulation of antibodies by immunization with heterologous or allogeneic antigens, but the technique can also apply to autoantibodies that arise without deliberate immunization. Autoantibodies that have been produced by hybridomas include those against erythrocytes (1), thymocytes (2), ribosomal RNA (3), DNA (4), and the Sm antigen (5). Heretofore, the method has been confined to mice with spontaneous systemic lupus erythematosus (e.g., NZB, B/W, and MRL-*lpr/lpr*). In our study we demonstrate the feasibility of producing human-human hybridomas that secrete autoantibodies, and we define conditions required for the derivation of stable autoantibody-producing clones from both spleen and peripheral blood lymphocytes of patients with autoimmune disease.

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## METHODS

**Human myeloma cell line.** The hypoxanthine phosphoribosyl transferase - deficient, hypoxanthine - aminopterin - thymidine(HAT)<sup>1</sup>-sensitive mutant line GM 4672, a subline of the GM 1500 cell developed by Croce et al. (6), was obtained from the Cell Repository, Institute of Medical Research, Camden, NJ.

**Isolation of lymphocytes.** Venous blood collected with preservative-free heparin was diluted 1:1 with RPMI 1640 containing 10 mM Hepes, 200 mM L-glutamine, 100 µg/ml penicillin-streptomycin, 1% pyruvate, and 0.5% nonessential amino acids (hereafter referred to as RPMI/Hepes). Spleen cells from the organ removed at splenectomy from a patient with immunothrombocytopenic purpura were prepared as a single cell suspension. Mononuclear cells from blood and spleen were harvested after gradient centrifugation with Ficoll-Hypaque and washed twice with RPMI/Hepes.

**Pokeweed mitogen (PWM) stimulation.** Mononuclear cells (10<sup>6</sup>) from either blood or spleen were incubated with a 1:100 dilution of PWM (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY). After 48 h the cells were washed twice with RPMI/Hepes and prepared for fusion.

**Fusion.** Mononuclear cells were fused with 10<sup>7</sup> GM 4672 cells in ratios of 5:1 and 10:1 by the polyethylene glycol (PEG) method (7). PEG 1540 (mol wt 1,300-1,600, J. T. Baker Chemical Co., Phillipsburg, NJ), was used at 23°C in concentrations of 38, 44, or 50% (vol/vol in RPMI 1640/Hepes). The mononuclear and GM 4672 cells were copelleted by centrifugation, and 2 ml of 38% PEG were added. The cell pellet was gently resuspended and then sedimented by centrifugation for 5 min at 200 g. In the case of 44% PEG, 0.5 ml of PEG was added to the cell pellet. The pellet was gently resuspended and then sedimented by centrifugation for 3 min at 300 g. In the case of 50% PEG, 0.5 ml of PEG was added to the cell pellet. The pellet was gently resuspended in the 0.5 ml of PEG, and after 1 and 3 min it was sequentially diluted by addition of 0.5 ml and then 1 ml of RPMI 1640/Hepes. The cells were then spun for 5 min at 200 g. After treatment of cells with PEG, the supernatants were aspirated, and the cells were resuspended in RPMI 1640/Hepes containing 15% fetal calf serum. After incu-

<sup>1</sup> **Abbreviations used in this paper:** ELISA, enzyme-linked immunoassay; HAT, hypoxanthine, aminopterin, thymidine; PBL, peripheral blood lymphocytes; PEG, polyethylene glycol; PWM, pokeweed mitogen; TBS, tris-buffered saline.

bation for 24 h (37°C, 5% CO<sub>2</sub>), the cells were pelleted, and the tissue culture fluid was replaced with HAT medium (8) supplemented with insulin (0.5 U/ml), oxaloacetic acid (0.132 mg/ml) and 5% NCTC 109 (Microbiological Associates, Walkersville, MD). The cells were seeded into either 0.2- or 2.0-ml wells of plastic culture plates (Costar, Data Packaging, Cambridge, MA) in a concentration of  $2 \times 10^5$  cells/well. The HAT medium was replaced every 5 d. 1 wk after hybrids were observed macroscopically (generally 4 wk after fusion), feeding was continued with hypoxanthine-thymidine medium. 1 wk later, the supernatants were tested for antibody production. Positive growths were cloned by limiting dilution in medium without hypoxanthine-thymidine in 0.2-ml wells of plastic culture plates. The cloned cells were fed every 7 d, and growth was usually visible after 2 wk. Subsequent cell culture was performed in vertical 75-ml flasks.

**Autoantibody screening. Anti-DNA antibodies.** An enzyme-linked immunoassay (ELISA) technique was used. Polystyrene plates with 96 flat-bottom wells (Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, VA) were coated first with poly-L-lysine (50 µg/ml), then with calf thymus ssDNA (2.5 µg/ml) prepared as described (9), and finally with poly-L-glutamate (50 µg/ml). Hybridoma supernatant (150 µl) was added to each well, and the plates were incubated for 1 h at room temperature. After washing with phosphate-buffered saline-Tween, 150 µl alkaline-phosphatase-conjugated goat anti-human immunoglobulin (IgG+IgM) was added. Plates were then incubated for 18 h and washed again. Bound alkaline-phosphatase conjugate was detected by addition of 150 µl *p*-nitrophenyl phosphate (1 mg/ml in 0.5 M NaHCO<sub>3</sub>, 2 mM MgCl<sub>2</sub>, pH 9.5) at 23°C. The reaction was stopped with 5 N NaOH, and optical densities were read at 405 nm in a Dynatech model MR580 Micro ELISA Reader.

**Antiplatelet antibodies.** Platelets obtained from citrated blood were fixed with 3% glutaraldehyde, washed twice with Tris-buffered saline, and stored in 60% glycerol at -20°C. Before use, the platelets were resuspended in 2 vol of Tris-buffered saline (TBS), sedimented at 500 *g* for 15 min, and resuspended in TBS at a concentration of  $0.5-1 \times 10^8$  cells/ml. A 100-µl aliquot of the platelet suspension was added to flat-bottom microtiter wells, and the platelets were sedimented by centrifugation at 1,000 *g* for 5 min. After aspiration of the supernatant, 200 µl of TBS with 0.5% gelatin was added to each well and the plate was incubated at 37°C for 30 min. The wells were washed thrice with TBS, hybridoma supernatant was added,

and the plate was incubated at 37°C for 60 min. After washing with TBS, 100 µl of alkaline phosphatase-conjugated goat anti-human immunoglobulin was added. The remainder of the assay was carried out as above.

**Cold agglutinin.** Hybridoma culture fluid (100 µl) was incubated with a 2% suspension of washed, ficin-treated, type O erythrocytes for 1 h at 4°C. The suspensions were examined for hemagglutination.

## RESULTS

Table I shows the yield of hybridomas from five fusions, four with peripheral blood lymphocytes and one with splenocytes, carried out under different conditions. Hybridomas appeared in 108/4,254 wells (2.5%), usually after 4 wk of incubation. Maintenance of the fused cells in 2.0-ml wells yielded hybridomas in 34/105 wells (32%), whereas incubation in 0.2-ml wells resulted in hybridomas in 74/4,149 wells (1.8%). Of the 108 hybridomas, 21 (19%) arose from fusions done with a PEG concentration of 38%; 72 (67%) with a PEG concentration of 44%; and 15 (14%) with a PEG concentration of 50%. A mononuclear/GM 4672 cell ratio of 5:1 produced 71 of the 108 hybridomas. Maximum yield of hybridomas (80/108) was obtained with lymphocytes that had been stimulated with PWM for 48 h before fusion. The single experiment that compared spleen cells with blood lymphocytes (F2 and F6) indicated that spleen cells were no more efficient in forming hybridomas than peripheral blood lymphocytes (1.2 and 1.1%, respectively).

Of the 108 hybridomas, 16 produced autoantibodies. All of them had the IgM isotype. Seven primary growths produced antibodies to ssDNA. All seven were cloned by limiting dilution, and in each case the procedure yielded multiple antibody-producing clones. Fig. 1A shows representative results of ELISA assays for ssDNA binding by supernatants of the cloned hybridomas. The IgG<sub>2</sub>κ protein produced by GM 4672

TABLE I  
Incidence of Hybridomas from Five Human Fusions under Various Conditions

Fusion No.	Diagnosis	No. clones	2-ml wells	0.2-ml wells	PEG concentration			Cell ratio		PWM stimulation	
					38%	44%	50%	5:1	10:1	with	without
F <sub>2</sub> (PBL)	SLE + ITP	11	—	11/900	4	5	2	7	4	10	1
F <sub>6</sub> (splenocytes)	SLE + ITP	15	—	15/1,280	2	11	2	10	5	15	0
F <sub>2</sub> (PBL)	Cold agglutinin disease	39	7/36	32/1,280	7	27	5	30	9	31	8
F <sub>1</sub> (PBL)	Atopic allergy	14	7/34	7/394	3	8	3	7	7	8	6
F <sub>2</sub> (PBL)	Atopic allergy	29	20/35	9/400	5	21	3	17	12	16	13
		108	34/105	74/4,254	21	72	15	71	37	80	28

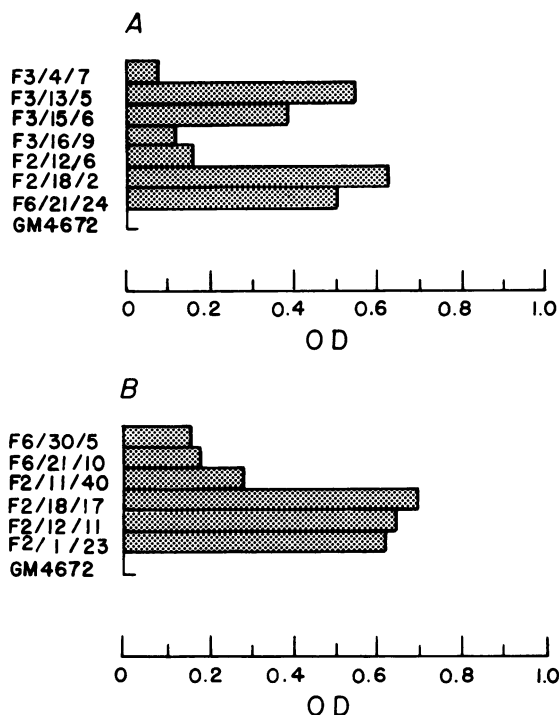


FIGURE 1 Results of direct binding ELISA assays of supernatants from representative cloned human-human hybridomas. (A) Binding to ssDNA; (B) binding to platelets.

cells failed to bind to ssDNA. Preliminary results of direct binding and competitive immunoassays (data not shown) indicated that all clones derived from a single primary growth gave identical or similar patterns of reactivity. Therefore, the primary growths were probably derived from single hybridomas.

Six primary wells contained antiplatelet activity and were also cloned by limiting dilutions. That procedure yielded 310 clones, 37 of which produced platelet-binding antibodies (Fig. 1B). The supernatants of the GM 4672 line had no antibody binding activity with platelets, nor did human-human hybrids derived from lymphocytes from a patient without autoimmune thrombocytopenia. As a positive control for the assay, human serum with anti-PL<sup>A</sup> antibodies contained antiplatelet activity, whereas normal serum had no activity.

Seven primary growths from the patient with cold agglutinin disease (F3) produced cold agglutinins. Limiting dilutions yielded 46 clones, of which 14 were positive for cold agglutinins. The supernatants of those clones were active at 4°C, but not at 27° or 37°C, and reactivity was revealed only with ficin-treated erythrocytes.

All of the cloned autoantibody-producing hybridomas have been passed in tissue culture repeatedly.

RPMI 1640/Hepes with 10% fetal calf serum has been used throughout. The oldest line has been passed for 7 mo, and it remains stable. The other lines also continue to produce autoantibodies except for 7/8 of the cold-agglutinin producing lines, which lost their ability to produce antierythrocyte antibodies. Passage in 75-ml or 150-ml vertical flasks has been done routinely. In such flasks the hybridomas are visible as nonadherent, macroscopic colonies, which form a carpet on the floor of the culture vessel.

## DISCUSSION

Our results demonstrate the feasibility of producing autoantibodies in tissue culture by means of human-human hybridomas. The GM 4672 cells we used are Epstein Barr nuclear antigen-negative,<sup>2</sup> and it is therefore unlikely that the clones we derived were Epstein Barr virus-transformed cells instead of hybridomas. Results with the GM 4672 line differ quantitatively from those obtained with mouse-mouse hybridomas. For instance, in the case of the hybridomas derived from spleen cells of MRL-*lpr/lpr* mice and the MPC-11 myeloma, the frequency of hybridomas ranged from 26 to 45% (4). By contrast, the corresponding figure in our experiments was 2.5%. Nevertheless, of 65 hybridomas obtained from the three human subjects with autoimmunity, the frequency of autoantibody-producing hybridomas was 16/65 (25%), a figure that compares favorably with the proportion in MRL-*lpr/lpr* mice (9–58% in various experiments (4)). The amount of autoantibody produced by the human-human hybridomas ranged from 1 to 15 µg IgM/ml (data not shown), whereas mouse-mouse hybridomas usually secrete 50–100 µg autoantibody/ml. Optimal conditions for obtaining hybridomas with the GM 4672 line include initial growth in 2.0-ml wells, the use of 44% PEG, a mononuclear cell/GM 4672 cell ratio of 5:1, and prior stimulation of the lymphocytes with PWM. The latter step, we believe, favors the fusion of autoantibody-forming B lymphocytes with GM 4672 cells. A PEG concentration of 50% was apparently toxic to some cultures, and we have abandoned its use. The difference between 44 and 38% PEG may not be statistically significant. Use of 2.0-ml wells may be advantageous by providing a relatively large surface area for growth of the hybridomas. In our experience, the ELISA technique readily measures the relatively low yield of autoantibody from human-human hybridomas. Moreover, we have found that cloned human-human hybridomas grow well in vertical 150-ml flasks, and by this means we have harvested as much as 600 ml of autoantibody-containing tissue culture fluid

<sup>2</sup> Thorley-Lawson, D. Personal communication.

from each clone. Affinity purification on DNA-Sepharose columns of fluids that contain anti-DNA antibodies can thus yield amounts of monoclonal autoantibody that are adequate for extensive analyses (unpublished observations). Thus far, our attempts to grow human hybridomas in nude mice have been unsuccessful.

Hybridoma technology is a means of obtaining pure monoclonal autoantibodies and it has been highly effective in murine systems. The present results indicate that a similar approach is possible with human autoantibodies. These monoclonal antibodies should greatly facilitate studies of the antigen specificity and the structure of the combining site of antibodies produced in SLE and other autoimmune disorders. The technique may also find applications in the study of allergic diseases that are mediated by immunoglobulins.

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