Human Monoclonal Antibody Against Forssman Antigen

Abstract. *Hybrid cells formed between human lymphocytes and mouse myeloma cells produce human immunoglobulin in culture. Stable antibody-producing cell lines can be isolated after multiple cycles of low-density passage, cloning, and continued selection for immunoglobulin production. The origin and characteristics of a hybrid of human and mouse cells is described. This hybrid produces high concentrations (8.3 micrograms per milliliter) of human immunoglobulin M reactive with the terminal disaccharide of the Forssman glycolipid. These findings point to the potential use of human-mouse hybrid cells as a source of human monoclonal antibodies for therapeutic and diagnostic purposes.*

The hybridoma techniques developed by Kohler and Milstein (*1*) provide a method for the production of virtually unlimited amounts of monoclonal antibodies. These antibodies have been used for the serological analysis of cell surface antigens (*2*) and viral antigens (*3*), and for the therapy of parasitic diseases (*4*) and malignant lymphomas (*5*) in mice. Although these studies with mouse monoclonal antibodies indicate their clinical potential, similar antibodies of human origin have yet to be described.

Two hybridization methods can be envisaged for the production of human monoclonal antibodies. The intraspecies method would involve the fusion of human lymphocytes to human myeloma cells, and the transspecies method would be performed between human lymphocytes and mouse myeloma cells (*6*, *7*). Since an appropriate human myeloma cell line is not yet available for the construction of intraspecies hybrids, we have evaluated the possibility of producing human monoclonal antibodies with transspecies hybrid cells formed by the fusion of human splenic lymphocytes to mouse myeloma cells. For this purpose, influenza virus was chosen as a model immunogen. We report success with this method and describe the preparation of a stable hybrid cell line (H1-C4) that continuously produces human monoclonal immunoglobulin M (IgM) antibody in culture. This antibody, with serological specificity for the Forssman glycolipid that is a constituent of the influenza virion, is produced in quantities comparable to those observed with conventional intraspecies mouse hybridomas.

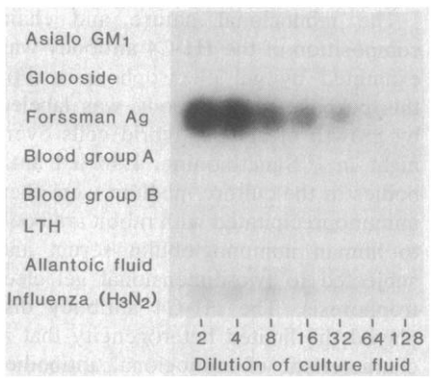

Fig. 1. Serological specificity of the H1-C4 monoclonal antibody. Culture fluids from the H1-C4 hybrid were tested in antibody-binding assays with a panel of glycolipids and related antigens. Immunoreactions were detected by autoradiography of the test plates for 16 hours at −70°C on Kodak NS-2T film with enhancement by x-ray intensifying screens. Abbreviations of glycolipids are as in (*19*): βGal-(1 → 3)-βGalNAc-(1 → 4)-βGal-(1 → 4)-βGlc-(1 → 1)-Cer (*Asialo GM*$_1$); αGal-(1 → 3)-βGal-(1 → 4)-βGlcNAc-(1 → 3)-βGal-(1 → 4)-βGlc-(1 → 1)-Cer (*Blood group B* glycolipid); and βGlcNAc-(1 → 3)-βGal-(1 → 4)-βGlc-(1 → 1)-Cer [(*LTH*), lacto-*N*-triosyl ceramide]. Structures of globoside, Forssman antigen (*Forssman Ag*), and blood group A^b glycolipids are given in Table 1.

Hybrid cell lines were derived by the polyethylene glycol (PEG)–mediated fusion of human lymphocytes to mouse (BALB/c) myeloma NSI/1 cells. The NSI/1 cell line (*8*) was an HPRT$^-$ (hypoxanthine phosphoribosyl transferase–lacking) variant that produced only a κ light chain. Human lymphocytes were obtained from the spleen of a 66-year-old female patient at surgery. The splenic cells were minced and gently suspended in Hanks saline solution and then, after washing by centrifugation, resuspended in tissue culture medium (RPMI 1640 supplemented with 15 percent fetal calf serum). To increase the probability of obtaining a reactive clone against the influenza virus, we adjusted the concentration of the spleen cells to 10^6 cells per milliliter and then stimulated them immunologically by incorporating influenza vaccine [Parke, Davis; containing hemagglutinin from influenza A (H_3N_2), influenza A (H_1N_1), and influenza B (each at a concentration of 14 μg/ml)] into the culture medium at a final concentration of 0.04 percent. Addition of this dose of influenza vaccine induced a mitogenic response (tenfold increased incorporation of [^{3}H]thymidine triphosphate compared to parallel cultures of spleen cells not incubated with vaccine). The lymphocytes were stimulated in vitro for 3 days, then washed twice in serum-free culture medium and fused to NSI/1 by centrifuging a mixture of both cell types (4:1 ratio of lymphocytes to NSI/1) into 40 percent PEG for 12 minutes at 250*g*. The PEG-treated cells were resuspended in HAT medium (hypoxanthine, aminopterin, and thymidine) and dispensed into Microtest II plates (10^6 cells per well). Viable hybrid cells were reproducibly obtained at a frequency of 10^{-5} to 10^{-6} of the human cells utilized.

Antibody products of the hybrid cells were assayed by antibody-binding assays in microtiter plates with ^{125}I-labeled protein A from *Staphylococcus aureus* (*9*). Influenza virus or purified glycolipids were used as antigens. For this purpose influenza viruses were propagated in chicken eggs and concentrated from allantoic fluid by centrifugation at 100,000*g* for 1 hour. Viral pellets were resuspended in phosphate-buffered saline (PBS), *p*H 7.2, containing 0.5 percent Nonidet P-40 and used as antigen preparations for the antibody-binding assay at a concentration of 20 μg/ml. Glycolipids were isolated and purified as previously described (*10*). In antibody-binding assays the glycolipids were used as antigens at a concentration of 20 μg/ml in water (*11*).

Hybrid cells were obtained in all 96 wells that were initially plated in the H1 fusion. Fourteen days after the wells were seeded, culture fluid from each of the wells was assayed in an antibody-binding assay with influenza virus (H_3N_2) or chicken allantoic fluid as antigen. Culture fluid from one of the wells (designated H1-C4) reacted specifically with the viral antigen, but not with the control antigen (allantoic fluid) or with the plastic wells to which antigen had not been ad-

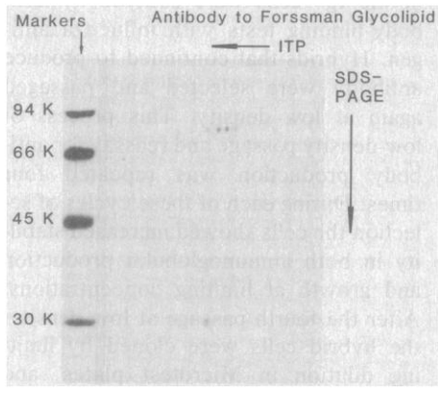

Fig. 2. Two-dimensional gel electrophoresis of the H1-C4 antibody. The [^{35}S]methionine-labeled immunoglobulins were immunoprecipitated from culture fluids with rabbit antibody to human immunoglobulin serum and then separated in one dimension by isotachophoresis (*ITP*) and in a second dimension by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (*SDS-PAGE*). Marker proteins (labeled with [^{3}H]dansyl chloride) included phosphorylase *a* (94,000 daltons), bovine serum albumin (67,000), ovalbumin (45,000), and carbonic anhydrase (30,000).

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Table 1. Structure of Forssman antigen and related glycolipids (*19*). Note that globoside is structurally identical to the Forssman glycolipid, except for its terminal sugar (GalNAc). Blood group A^b glycolipid contains the same terminal GalNAc residue as the Forssman glycolipid, but differs at structures in the penultimate position.

Glycolipid	Structure
Globoside	βGalNAc-(1 → 3)-αGal-(1 → 4)-βGal-(1 → 4)-βGlc-(1 → 1)-ceramide
Forssman	αGalNAc-(1 → 3)-βGalNAc-(1 → 3)-αGal-(1 → 4)-βGal-(1 → 4)-βGlc-(1 → 1)-ceramide
Blood group A^b	αGalNAc-(1 → 3)-βGal-(1 → 4)-βGlcNAc-(1 → 3)-βGal-(1 → 4)-βGlcNAc-(1 → 3)-βGal-(1 → 4)-βGlc-(1 → 1)-ceramide ↑2 L-αFuc-1

sorbed. When the hybrids from the H1-C4 well were semiconfluent, they were transferred into new Microtest II plates. Since the cloning efficiency of these cells was relatively low (< 5 percent) soon after fusion, this initial transfer was performed at limiting dilutions in the presence of feeder cell suspensions of BALB/c mouse thymocytes. These cell mixtures contained hybrid cells at a concentration of 50 cells per milliliter and thymocytes at a concentration of 3.5×10^6 cells per milliliter. Two hundred microliters of the cell suspension were plated into each well to attain a final seeding of ten hybrid cells per well. This process of low-density passage enabled the separation of immunoglobulin-producing (Ig^+) hybrids from the majority of hybrids that phenotypically converted to the nonproducing (Ig^-) phenotype. This was of particular importance, since the Ig^- cells had a selective growth advantage over the Ig^+ cells and tended to overgrow in cultures of mixed cell populations.

Culture fluids from the second passage of the Ig^+ cells were assayed by antibody-binding tests with influenza antigen. Hybrids that continued to produce antibody were selected and passaged again at low density. This process of low-density passage and reassay for antibody production was repeated four times. During each of these cycles of selection the cells showed increased stability in both immunoglobulin production and growth at limiting concentrations. After the fourth passage at low density, the hybrid cells were cloned by limiting dilution in Microtest plates, and their culture fluids were tested for specific antibody. Immunoglobulin-producing clones were then selected, and the cloning cycle was repeated twice. At each cloning cycle a minimum of 100 daughter clones was examined for antibody production. The percentage of antibody-producing clones improved with each cycle, so that by the third cycle of cloning more than 95 percent of the daughter clones were producing high-titered antibody. After continuous passage in culture, the growth properties of the hybrid cells stabilized and the addition of feeder cells was not necessary. Antibody production in these cultures has now persisted for more than 7 months.

Specificity of the H1-C4 antibody was determined by antibody-binding assays with a panel of five serologically distinct isolates of influenza virus. The results of these tests showed that the H1-C4 antibody reacted with three of the five serotypes of influenza virus (H_1N_1, H_3N_2, and B). Specificity of these reactions was assured by the nonreactivity of the antibody with two other serotypes of influenza (H_0N_1 and H_2N_2) and with allantoic fluid. Although these patterns of activity showed that the H1-C4 antibody detected an influenza-related antigen, the cross-reaction observed between A and B strains of influenza indicated that the antigen was neither hemagglutinin nor neuraminidase.

On the possibility that the antigen represented a nonprotein constituent of the virus, the H1-C4 antibody was tested further for reactivity with a panel of different glycolipids. The antibody reacted strongly with purified Forssman glycolipid, as well as with influenza virus (Fig. 1). The antibody did not react with five other purified glycolipids or with allantoic fluid. An analysis of the structural relationships among these glycolipids (Table 1) indicated that the H1-C4 antibody was reactive with the terminal disaccharide [αGalNAc-(1 → 3)-βGalNAc-(1 → R)] (*12*) of the Forssman glycolipid (*13*). In fact, the specificity of this antibody parallels that observed with antibodies to Forssman glycolipid found naturally in human serums (*14*) and in a human monoclonal antibody to Forssman glycolipid obtained from a patient with Waldenström macroglobulinemia (*15*). This observation is also consistent with the fact that influenza virus and influenza vaccine both contain a number of glycolipids, including the highly immunogenic Forssman antigen and blood group A substances (*16*).

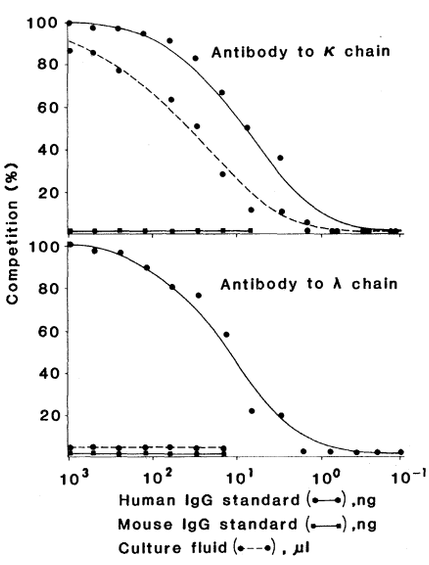

Fig. 3. Light chain composition of the H1-C4 antibody. Competition RIA (*7*) was performed with culture fluid with H1-C4 cells. Control samples included purified human IgG and purified mouse IgG. Antiserums included rabbit antibody to human κ chain and rabbit antibody to human λ chain.

The monoclonal nature and chain composition of the H1-C4 antibody was examined by gel electrophoresis. For this purpose, the antibody was labeled by growth of H1-C4 hybrid cells overnight in [^{35}S]methionine. Labeled antibodies in the culture medium were then immunoprecipitated with rabbit antibody to human immunoglobulin serum and subjected to two-dimensional gel electrophoresis. The H1-C4 antibody displayed the limited heterogeneity that is characteristic of monoclonal antibodies (*9*) (Fig. 2). A single light chain and a heavy chain (approximately 80,000 daltons), containing multiple charge variants, were observed. This electrophoretic profile is similar to that observed with IgM monoclonal antibodies (*9*). The light chain composition of the monoclonal antibody was investigated further by competition radioimmunoassay (RIA). The H1-C4 culture fluid contained human κ light chain at a concentration of 8.3 μg per milliliter of culture fluid (Fig. 3). Analogous RIA assays performed with heavy chain–specific reagents showed that H1-C4 is a human IgM. The

hybrid nature of the H1-C4 cell line was confirmed by karyotypic analysis. The H1-C4 cells contained between 129 and 133 chromosomes. Of these chromosomes, 10 to 12 were identified as human metacentric chromosomes, one was identified as the metacentric marker chromosome of the NSI/1 cell, and the rest were acrocentric chromosomes typical of mouse cells.

The production in culture of high-titered human antibodies by transspecies hybrid cells raises the question of whether this technique will have broad application. Although the potential for this method has been recognized (*6, 7*), efforts at developing transspecies hybrids have been hindered by observations that these cells have a high rate of chromosome loss and phenotypic instability. We find, however, that if hybrid cells can be generated at a sufficient frequency, then aggressive selection techniques can compensate for the high loss rate and permit the isolation of the minority population of stable hybrid cells.

Although the majority of our transspecies hybrids were initially phenotypically unstable (transspecies fusions produced 1 to 10 percent as many stable hybrids as intraspecies mouse fusions did), we have now obtained six different stable hybrid cell lines that each produce human monoclonal IgM antibodies. In all of these instances, more than 80 percent of the daughter clones from individual hybrid cell lines showed continued IgM production in culture. Furthermore, several of these stable antibody-producing hybrids have been obtained from fusions that were performed between mouse myeloma cells and nonstimulated human lymphocytes from blood and lymph nodes. It appears therefore that in vitro stimulation with antigen, although useful for the expansion of reactive B cell clones, may not be essential for the generation of successful hybrids.

With increased developments in methods for the selection of antibody-producing hybrids [for example, by use of the fluorescence-activated cell sorter (*17*)] it should be possible to use transspecies hybridization as a general method for the production of human monoclonal antibodies for therapeutic and diagnostic purposes. In this context, studies by Hakomori *et al.* (*18*) have demonstrated the "tumor-specific" expression of Forssman glycolipid in gastric tumors of patients who were apparently Forssman-negative. The selective expression of this glycolipid on tumor cells provides a potential target for immunological intervention. The availability of the H1-C4 human monoclonal antibody against Forssman antigen now permits a more detailed examination of this possibility.

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12. Abbreviations: Gal, galactose; GalNAc, *N*-acetylgalactosamine; R, a substrate; Glc, glucose; GlcNac, *N*-acetylglucosamine; Fuc, fucose; and Cer, ceramide.
13. The reactivity of H1-C4 antibody with Forssman antigen was further confirmed by hemagglutination and hemolysis assays. H1-C4 antibody both agglutinated and lysed sheep red blood cells; in parallel assays the antibody did not react with guinea pig or human red blood cells.
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Antibody Targeting of Liposomes: Cell Specificity Obtained by Conjugation of F(ab')$_2$ to Vesicle Surface

Abstract. *A method devised for conjugating liposomes with protein resulted in the binding of up to 200 micrograms of immunoglobulin G per micromole of lipid. The coupling of antibody to human erythrocyte F(ab')$_2$ in vesicles (140 molecules per vesicle) by this method caused a 200-fold increase in the binding of vesicles to human erythrocytes and resulted in about 80 percent of the vesicle lipid and contents being associated with cells.*

Liposomes have gained wide acceptance as potential carriers for introducing drugs and macromolecules into cells (*1*). Several investigators have attempted to increase the extent of liposome interaction with specific cells by inducing specific ligand receptor interactions. Such attempts have included noncovalent association of cell-specific antibodies with liposomes (*2, 3*), coating of liposomes with heat-aggregated immunoglobulin M (IgM) (*4*), lectin-mediated attachment of glycoprotein-bearing liposomes with erythrocytes (*5*), and use of hapten-bearing liposomes and antibody to haptens (*6, 7*). Only two- to sixfold increases in binding were obtained by these methods and a small fraction of the liposomes (up to 5 percent) were affected. Nonspecific association of antibody to vesicles typically involves only 1 to 6 μg of protein per milligram of lipid, and previous attempts to enhance binding by covalent attachment with glutaraldehyde or carbodiimide (*8*) achieved little success. We reported recently a procedure for the attachment of periodate oxidized horseradish peroxidase to liposomes bearing primary amino groups by imine formation and borohydride reduction (*9*). We have now devised an efficient coupling method applicable to many proteins, in which aldehydes on the vesicle surface react with amino groups on the protein (*10*). The aldehyde groups are produced by periodate oxidation of vesicles containing lipids bearing vicinal hydroxyls (glycosphingolipids). With this method up to 200 μg of immunoglobulin G (IgG) is bound per micro-

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