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*J Clin Invest.* 1982;70(6):1306-1309. <https://doi.org/10.1172/JCI110730>.

**Research Article**

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# Protective Human Hybridoma Antibody to Tetanus Toxin

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**ABSTRACT** Postimmunization human B lymphocytes and mouse myeloma cells were fused to produce interspecies hybridomas secreting human antibody of predefined specificity with an initial frequency comparable to intraspecies fusion. After 13 mo in culture, one clone continued to secrete high titers of human IgG antitetanus toxin antibody. This antibody binds to the B fragment of tetanus toxin and protects mice against tetanus. The demonstration of *in vivo* protection with a human monoclonal antibody is an important first step towards the ultimate goal of human administration of monoclonal antibodies for the prevention and therapy of human infections.

## INTRODUCTION

Monoclonal antibodies produced by hybrid cell lines have potential use in the therapy of human infection (1), malignancy (2), and transplantation rejection (3). However, human rather than murine monoclonal antibodies will be required to avoid sensitization to foreign proteins with their *in vivo* application (4). As yet, protective human monoclonal antibodies have not been described and the optimal method for the production of human hybridoma antibodies is yet to be determined. Only limited success has been reported using human myeloma cell lines as fusion partners to immortalize antibody-producing lymphocytes (5, 6). The rapid loss of human chromosomes from mouse myeloma-human lymphocyte hybrid cell lines has hampered the production of human antibody by this technique, although a few interspecies hybrids have been reported (7-9). One possible way to circumvent the instability of interspecies hybrids would be to increase the number of hybrids initially secreting an antibody of predefined specificity, so that more attempts could be made at cloning a stable line. We sought to accomplish this by immunizing volunteers with tetanus toxoid and then harvesting their peripheral

blood B lymphocytes for fusion at a time correlating with peak *in vitro* production of antitetanus toxoid antibody. Tetanus toxin was chosen as a model antigen because of its potent immunogenicity in man, the availability of a mouse toxin neutralization assay, and because human tetanus immunoglobulin is currently used in both the prevention and therapy of human tetanus.

This report describes production of a stable hybrid cell line that secretes a human IgG antibody by fusing postimmunization human peripheral blood lymphocytes with the nonsecretor mouse myeloma line P3-X63-Ag 8.653 (10). This monoclonal antibody (9F12) binds tetanus toxin *in vitro* and prevents tetanus *in vivo* in animals. The antigenic specificity of this antibody is described.

## METHODS

**Production of human monoclonal antibody.** Peripheral blood mononuclear cells were obtained by Ficoll-Hypaque gradient centrifugation of heparinized peripheral blood collected from volunteers 6 or 7 d after immunization with tetanus and diphtheria toxoids adsorbed (for adult use) (Wyeth Laboratories, Marietta, PA). No volunteer had received a tetanus immunization within the preceding 2 yr and the individual from whom monoclonal antibody 9F12 was derived was last immunized ~10 yr earlier. An enriched B lymphocyte fraction was prepared by incubating the mononuclear cells with 2-aminoethylisothiouonium bromide-treated sheep erythrocytes followed by Ficoll-Hypaque gradient centrifugation to remove lymphocytes rosetting with the erythrocytes (11). The enriched B cells were fused with an equal number of P3-X63-Ag 8.653 mouse myeloma cells, according to a standard fusion protocol (12), using 50% polyethylene glycol 6000 (Fisher Scientific Co., Fairlawn, NJ) as the fusing agent. After fusion, the cells were maintained in RPMI 1640 media containing 15% fetal calf serum,  $2 \times 10^{-3}$  M glutamine,  $1 \times 10^{-3}$  M sodium pyruvate, 100 U/ml penicillin, 100  $\mu$ g/ml gentamicin,  $1 \times 10^{-4}$  hypoxanthine,  $1.6 \times 10^{-5}$  M thymidine (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY),  $4 \times 10^{-7}$  M aminopterin (Sigma Chemical Co., St. Louis, MO),  $5 \times 10^{-5}$  M 2-mercaptoethanol (Eastman Kodak Co., Rochester, NY), 5  $\mu$ g/ml insulin and 5  $\mu$ g/ml transferrin (Collaborative Research, Lexington, MA). The cells were cultured at a density of  $1.5 \times 10^5$  cells/0.2 ml in microtiter wells (Costar 3596, Costar Data Packaging, Cambridge, MA) that contained

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Dr. Gigliotti is a Wilmut Cancer Fellow.

Received for publication 30 July 1982 and in revised form 1 September 1982.

$3 \times 10^3$  irradiated mouse peritoneal macrophages as a feeder layer.

The hybrid supernatants were screened for antitetanus toxoid antibody production using an enzyme-linked immunosorbent assay (ELISA)<sup>1</sup> in microtiter plates (Microbiological Associates, Walkersville, MD) preincubated with 100  $\mu$ l of purified tetanus toxoid (Massachusetts Public Health Biologic Laboratories, Boston, MA) at 10  $\mu$ g/ml. Antibody binding to tetanus toxoid was identified with alkaline phosphatase-labeled antisera specific for human immunoglobulin (Ig)G, IgA, and IgM (Atlantic Antibodies, Scarborough, ME) prepared and tested for specificity as has been previously described (13). ELISA assays were also performed with diphtheria toxoid (Massachusetts Public Health Biologic Laboratories), at 1  $\mu$ g/ml.

Antibody-secreting hybrids were passed several times at *d*-10 cells/microtiter well that contained a mouse macrophage feeder layer. After five of these low density passages, hybrid 9F12 was cloned twice in a limiting dilution assay at 1 cell/well (14).

**Characterization of 9F12.** The antibody from the tissue culture supernatant was concentrated ~10-fold by precipitation with an equal volume of saturated ammonium sulfate (SAS) followed by dialysis against phosphate-buffered saline (PBS). Immunoglobulin heavy and light chain were determined by Ouchterlony immunodiffusion using goat anti-human and anti-mouse antisera (N. L. Cappel Laboratories, Cochranville, PA). The concentration of antibody in tissue culture supernatant allowed to overgrow for several days was determined by radial immunodiffusion using an LC-partigen IgG kit (Calbiochem-Behring Corp., La Jolla, CA).

**Determination of the antigenic specificity of 9F12.** Purified tetanus toxin was radioiodinated by the lactoperoxidase method using Enzymobeads (15) (Bio-Rad Laboratories, Richmond, CA). The iodinated toxin (1  $\mu$ Ci/ $\mu$ g) was incubated with papain (Boehringer Mannheim Biochemicals, Indianapolis, IN) at a ratio of 2 U papain/mg toxin for 3 h at 55°C as previously described (16). SAS concentrated hybridoma supernatant and human serum were then incubated with the papain digest for 1.5 h at 4°C and antigen-antibody complexes were precipitated with formalin-fixed *Staphylococcus aureus*, strain Cowan 1 (Enzyme Center, Inc., Boston, MA). The precipitate was washed three times with PBS and prepared for SDS-polyacrylamide gel electrophoresis by vortexing the pellet with SDS sample buffer, incubating for 5 min at 20°C, centrifuging the sample and then boiling the supernatant for 2 min before applying it to a 7.5% SDS-polyacrylamide gel. The gel was then fixed and autoradiography performed with Kodak X-Omat AR film.

**Mouse toxin neutralization assay.** The biologic activity of 9F12 was investigated using the mouse tetanus toxin neutralization assay (17). Purified tetanus toxin (lot 39, Massachusetts Public Health Biologic Laboratories) in PBS with 0.2% gelatin (PBS-G) was diluted with an equal volume of either PBS-G, 10-fold SAS concentrated mouse myeloma cell tissue culture supernatant (653), or 10-fold SAS concentrated human monoclonal antibody 9F12 and administered as a 0.5-ml subcutaneous injection to the right inguinal fold of 15-18-g female CD-1 mice (Charles River Laboratories, Wilmington, MA). The minimal lethal dose of this toxin preparation, defined as 100% death by 96 h, was 0.16 ng.

## RESULTS AND DISCUSSION

B lymphocytes prepared for fusion were harvested on days 6 or 7 postimmunization, which in preliminary studies corresponded to the time of peak *in vitro* antitetanus toxoid antibody production, confirming previously published data (18). In the fusion from which clone 9F12 was derived,  $6 \times 10^6$  B lymphocytes were initially seeded into 80 microtiter wells. Hybrids were observed in 28 of the 80 wells by 3 wk after fusion. 5 of the 28 wells contained human antitetanus toxoid antibody, all of the IgG isotype. Four of the five wells were passed at 10 cells/well and reassayed for continued antibody production. After several cycles of this procedure, one clone, 9F12, continued to produce high titers of antibody of the IgG class with k light chain (Fig. 1). No mouse immunoglobulin was detectable. Karyotyping performed after the second cloning by limiting dilution assay showed the cells to have a modal chromosomal number of 108 with ~101 mouse and 6-7 intact human chromosomes. Antibody production measured 4 mo after fusion was ~2.5  $\mu$ g/ml. Repeat determination 4 mo later and after successful cloning showed antibody production to be slightly increased at 5-10  $\mu$ g/ml.

Following the same protocol, B lymphocytes from three other immunized volunteers were fused and seeded into a total of 384 wells. Viable hybrids grew in 94 of the wells and 5 of these secreted human antitetanus toxoid antibody of the IgG isotype. Low den-

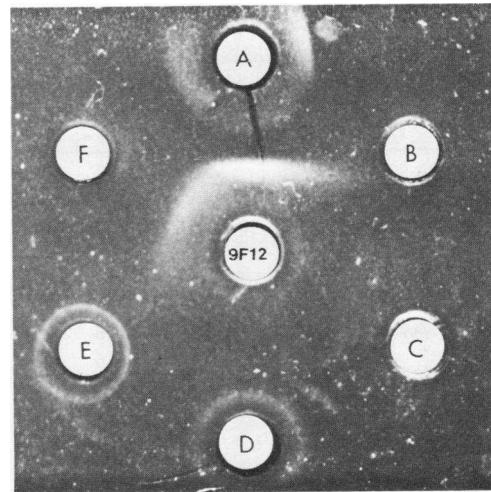


FIGURE 1 Ouchterlony immunodiffusion pattern of human monoclonal antibody to tetanus toxin (9F12). The outer wells contain the following goat antisera: anti-human IgG(A), anti-human IgA(B), anti-human IgM(C); anti-mouse IgM, IgG, and IgA(D), anti-human  $\lambda$ -light chain (E) and anti-human k light chain (F).

<sup>1</sup> Abbreviations used in this paper: ELISA, enzyme-linked immunosorbent assay; SAS, saturated ammonium sulfate.

sity passage was attempted on three of the secreting hybrids but we were unable to maintain antibody production for >1 mo. Human antidiphtheria toxoid antibody was detected in supernatants of 3 of the 122 hybrids. No supernatants reacted with both tetanus and diphtheria toxoids.

We were able to demonstrate the protective capacity of this human hybridoma antibody using the mouse tetanus toxin neutralization assay. Data from a representative experiment are shown in Table I. All 15 animals that received tetanus toxin neutralized by preincubation with antibody 9F12 were completely protected. On the other hand, all 34 animals that received toxin preincubated with PBS-G, or the parent mouse myeloma cell culture supernatant, were either killed or paralyzed by the toxin. These results were reproducible in four replicate determinations. To the best of our knowledge, this is the first demonstration of toxin neutralization by a hybridoma antibody. The possibility does exist that there could be a mutant tetanus toxin which would not be neutralized by monoclonal antibody 9F12. However, mutant forms of tetanus toxin have never been described.

Tetanus toxin has a molecular weight of ~150,000 and consists of two polypeptide chains linked by a disulfide bond. With reduction, the extracellular form of the toxin is split into its constituent heavy and light chains. Papain digestion cleaves the molecule into a 47,000-mol wt fragment C and an ~95,000-mol wt fragment B (16). Fragment C consists of the carboxy terminal half of the heavy chain and is responsible for

TABLE I  
Mouse Tetanus Toxin Neutralization Assay Using Human Monoclonal Antibody as Antitoxin

Toxin dose	No. dead/No. total at indicated time after toxin administration					
	24	48	72	<sup>h</sup> 96	120	144
0.16 ng in PBS-G*	0/9	0/9	8/9	9/9	—	—
0.16 ng in 653*	0/5	0/5	4/5	5/5	—	—
0.16 ng in 9F12†	0/5	0/5	0/5	0/5	0/5	0/5
0.08 ng in PBS-G	0/5	0/5	0/5	1/5	4/5	5/5
0.08 ng in 653	0/5	0/5	0/5	2/5	4/5	5/5
0.08 ng in 9F12	0/5	0/5	0/5	0/5	0/5	0/5
0.04 ng in PBS-G	0/5	0/5	0/5	0/5	0/5	0/5
0.04 ng in 653	0/5	0/5	0/5	0/5	0/5	0/5
0.04 ng in 9F12	0/5	0/5	0/5	0/5	0/5	0/5

\* All animals receiving 0.16 or 0.08 ng of toxin in PBS-G or 653 died by 6 d, while those receiving 0.04 ng survived but had right-sided paralysis.

† All three groups receiving 9F12 showed no clinical signs of tetanus.

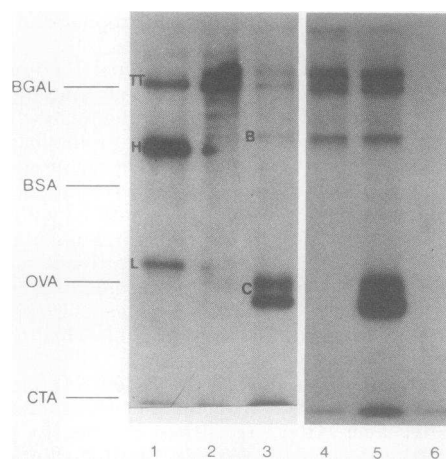


FIGURE 2 SDS-polyacrylamide gel electrophoresis of papain digested <sup>125</sup>I-labeled tetanus toxin (TT) after incubation with 9F12 or heterologous antisera (FG) and precipitation with *S. aureus*, strain Cowan 1. TT was reduced by boiling the sample in 5% 2-mercaptoethanol for 2 min before loading the sample on the gel. Lanes contain the following: 1-reduced TT, 2-nonreduced TT, 3-TT digested with papain, 4-TT digested with papain and incubated with 9F12, 5-TT digested with papain and incubated with fetal calf serum, 6-TT digested with papain and incubated with fetal calf serum. H, heavy chain, L, light chain, B, fragment B, C, fragment C. TT and fragment C may appear as a doublet or triplet in SDS-polyacrylamide gels. The tetanus toxin has not been completely digested with papain. Molecular weight markers were  $\beta$ -galactosidase (BGAL), bovine serum albumin (BSA), ovalbumin (OVA) and chymotrypsinogen A (CTA).

the binding of the toxin to its *in vivo* receptor (19). Fragment B consists of the remaining portion of the heavy chain linked to the entire light chain. Although the function of fragment B is unknown, it is hypothesized to contain the toxin principle of the molecule (20).

Hybridoma antibody 9F12 binds both intact tetanus toxin and toxin fragment B (Fig. 2). Serum (FG) from the individual whose lymphocytes were used in the fusion producing 9F12 bound both fragments B and C as well as the intact toxin. Other experiments (data not shown) demonstrated that 9F12, in contrast to the FG serum, is unable to bind purified toxin heavy or light chains that were obtained by treatment of tetanus toxin with dithiothreitol and urea and isolation of the chains by gel filtration as described (21). This would suggest that the antigenic specificity of 9F12 is directed to either a conformational epitope requiring the disulfide linkage of the heavy and light chain or an epitope on one of the chains that was denatured with reduction and dissociation of the toxin. The actual mechanism of toxin neutralization by the monoclonal antibody is under investigation.

The advent of monoclonal antibody technology has rekindled an interest in the passive immunotherapy of various human diseases. However, up to this point, most monoclonal antibodies have been of mouse origin and protective human monoclonal antibodies have not been reported. This report demonstrates that it is possible to produce monoclonal antibodies of human origin with a specifically desired biologic function using readily accessible human lymphocytes. Specific antibody secretion occurred in 11% (13/122) of the mouse-human hybrids using postimmunization human lymphocytes, which is comparable to results obtained with intraspecies fusions. Recently, other investigators have also been successful in producing mouse-human fusions after *in vivo* immunization (9). The protective capacity of 9F12 against tetanus demonstrates that a monoclonal antibody can neutralize this toxin and supports the prospect that human monoclonal antibody will have a role in the prevention and treatment of human infection.

#### ACKNOWLEDGMENTS

We thank Ann Kittelberger for technical assistance and Larry Wisniewski for karyotyping the hybrid cell line.

This work was supported in part by National Institutes of Health grant AI 17217 and by U. S. Public Health Service grant RR 05403-BRSG.

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