THE PRODUCTION OF A BISPECIFIC ANTI-CEA, ANTI-HAPTEN (4-AMINO-PHTHALATE) HYBRID-HYBRIDOMA

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A standard hybridoma fusion technique was used to produce a monoclonal antibody capable of binding both carcinoembryonic antigen (CEA) and the hapten 4-amino-phthalate. A hypoxanthine-aminopterin-thymidine (HAT) sensitive anti-CEA hybridoma and KLH-phthalate immunized spleen cells were hybridized to yield clones producing bispecific monoclonal antibodies. The desired bispecific antibody was identified using both enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay. The resultant hybrid-hybridoma or "tridoma" was subcloned and expanded to yield a stable population. Bifunctional antibody was then isolated from the various possible recombinants by ion exchange chromatography. This general method may be used to produce bispecific monoclonals against a wide variety of tumor associative antigens and reagents for immunodetection or treatment. (*J Natl Med Assoc.* 1991;83:901-904.)

Key words • anti-CEA hybridoma • 4-amino-phthalate • monoclonal antibody • bispecific antibody • immunotargeting

Immunotargeting using radiolabeled polyclonal antibody was first completed in vivo by Pressman and Korngold in 1953.[1] After removal by absorption of cross-reacting antibodies, specificity to tumor was demonstrated. Greater selectivity against tumor antigens became possible with the introduction of monoclonal antibodies by Kohler and Milstein.[2]

Antitumor monoclonals have been of limited success when used alone as passive immunotherapy for treating tumors.[3-5] Current clinical trials are exploring the use of monoclonals as delivery agents for toxins,[6] chemotherapeutic drugs,[7] cytotoxic lymphocytes,[8,9] and radiolabels.[10,11] Several chemical methods of conjugating monoclonals and functional reagents have been described,[12,13] but harsh conditions that can result in low yields, protein aggregation, or a loss of immunoreactivity are often required. An alternate method uses standard hybridoma fusion techniques to link monoclonal antibody with drugs or other reagents, thus avoiding harsh chemical conditions.[14]

Fusing two antibody-producing cell lines can produce a cell that secretes an antibody with binding characteristics similar to the parental monoclonals. A single antibody is thus capable of monovalent binding between tumor antigens and drugs or toxins (Figure 1). Once such an antibody has accumulated within a tumor, reagents can be specifically targeted to the tumor using the second binding site to capture and localize the reagent. This study used spleen cells from hapten (4-amino-phthalate) immunized mice as a fusion partner with a hypoxanthine-aminopterin-thymidine (HAT) sensitive anti-carcinoembryonic antigen (anti-CEA) hybridoma. The resulting hapten can be conjugated to multiple reagents for localization to CEA-producing tumors so that individual hybrid-hybridomas do not need to be constructed for each drug, toxin, or radiolabel.

MATERIALS AND METHODS

Selection of HAT Sensitive Anti-CEA Hybridoma

An anti-CEA hybridoma (8019) was obtained from the American Type Culture Collection and cultured in RPMI 1640 with 10% fetal calf serum. Hypoxanthine-aminopterin-thymidine sensitivity was selected by culturing in the presence of 8-azoquanine at an initial concentration of 5 μg/mL; final concentration was 20 μg/mL. Those clones sensitive to HAT media were backselected and used as fusion partners. Supernatants were periodically checked for continued CEA antibody production using enzyme-linked immunosorbent assay (ELISA).

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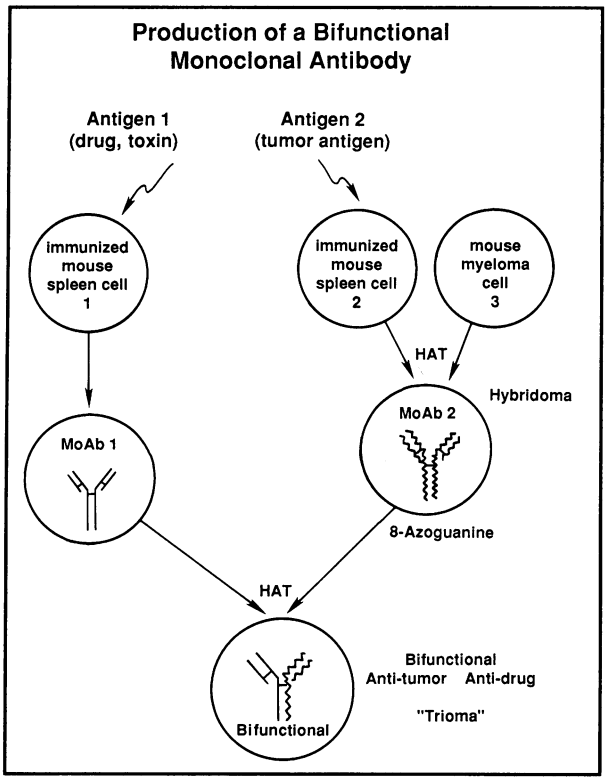

Figure 1. Hybrid-hybridoma for the production of bifunctional monoclonal antibody.

Anti-Phthalate Production

4-amino-phthalate was diazotized and coupled to keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) at a 0.5-mm salt per gram protein.[15] Adult female Balb-c mice were immunized subcutaneously with weekly phthalate-KLH (200 μg) in Freunds adjuvant. After 3 weeks, serum levels were monitored for anti-phthalate production using a solid phase ELISA. Spleens from positive responders were pooled and used as fusion partners. Phthalate-BSA was radiolabeled using the iodogen method for use in the radioimmunoassay.

An anti-phthalate hybridoma (IgB6) was obtained from Roswell Park Memorial Institute. The supernatant from IgB6 was used in the radioimmunoassay to measure maximum binding of the radiolabeled phthalate—BSA.

Fusion and Antibody-Binding Assays

Spleenocytes from immunized mice were fused using polyethylene glycol with HAT sensitive 8019 hybridomas at a ratio of 20:1 according to the method of Gefter et al.[16] Supernatants were tested 2 weeks after fusion for antibody production against both CEA and phthalate.

Target plates for anti-CEA were prepared by fixing confluent CEA expressing cell lines—RWP1.1, ASPC1, and BXPC3 in 96 well plates with 0.5% glutaraldehyde for 24 hours at 4°. Plates were washed with 0.5% polysorbate (Tween) and blocked with 0.3% BSA. Plates were kept frozen in 0.3% BSA and thawed prior to use.

Carcinoembryonic antigen was also obtained from perchloric acid extracts of human gastrointestinal tract tumors and absorbed onto flexible 96 well plates. Western blots using anti-CEA monoclonal confirmed immunoreactive CEA in the extracts.

Bovine serum albumin-phthalate (20 μg) was absorbed onto polyvinyl chloride plates and used to monitor anti-phthalate production. Supernatant was tested by incubating 25 μL on each of the target plates at 37°C for 2 hours. Plates were then washed with 0.05% polysorbate and rinsed with distilled water. A peroxidase conjugated rabbit antimouse antibody (Dako-p161) 1:200 in 0.3% BSA was applied for 1 hour followed by a distilled water rinse ×5. Substrate consisted of o-phenylenediamine (20 mg/50 mL citrate buffer, pH 5.0) with 10 μL hydrogen peroxide 100 μL/well. The reaction was terminated with 50 μL 3M sulfuric acid. Absorbance was read at 492 nm. Optical density values greater than 0.3 OD were considered positive.

Radioimmunoassay for Bispecificity

Supernatant (20 μL) was incubated on CEA-antigen coated plates for 2 hours then blocked with 0.3% BSA and washed ×5 with 0.05% polysorbate. I^{125} BSA-phthalate was added to each well for 1 hour followed by a repeat wash. Individual wells were dried, and bound radioactivity measured in a gamma counter. Maximum binding was determined by using supernatant from the established anti-phthalate hybridoma instead of CEA to coat wells followed by radiolabeled BSA-phthalate. Negative controls substituted IgB6 supernatant for the bispecific monoclonal on CEA target plates.

DEAE Column Chromatography

A DEAE column (0.5 × 6 cm) was equilibrated with 10 mm NaCl (pH 7.0) and loaded with ammonium precipitated supernatant that had been dialyzed against the starting buffer. Protein was eluted with a linear gradient of NaCl (10 mm-100 mm). The effluent was monitored using both CEA and BAS-phthalate target plates and absorption at 280 nm.

RESULTS

Two weeks following fusion, 504 wells were

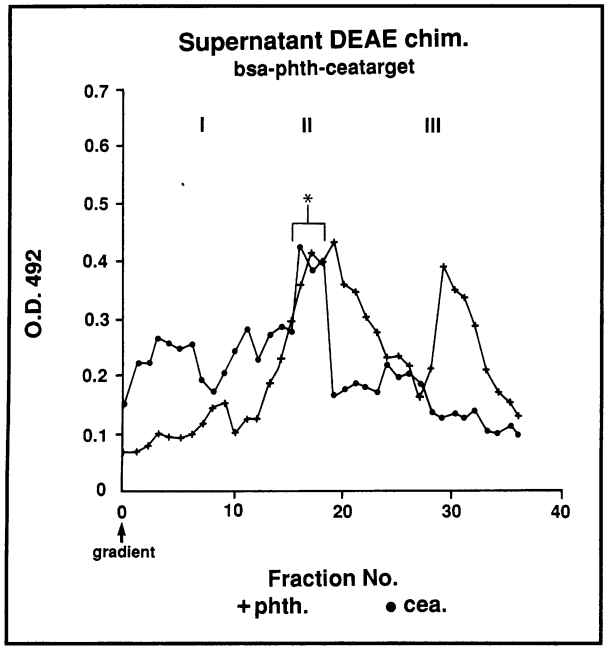

Figure 2. DEAE chromatography of supernatant. Fraction II exhibited dual specificity to both CEA and BSA-phthalate.

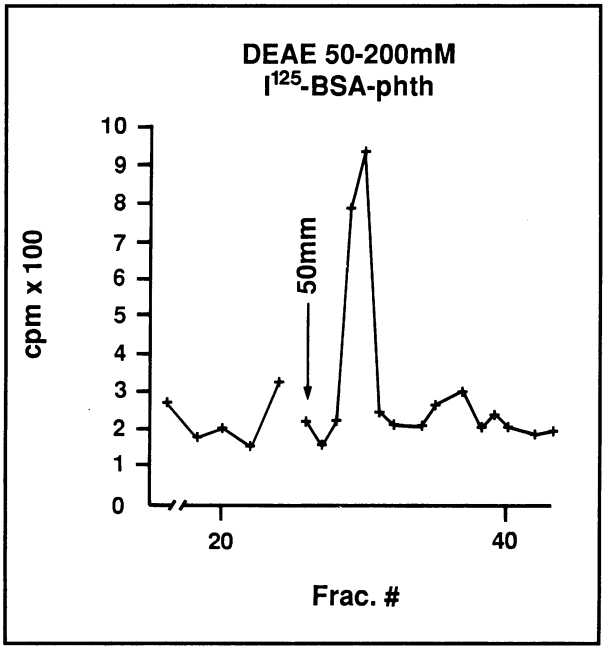

Figure 3. DEAE chromatography of fraction II. Fractions incubated on CEA target plates followed with I^{125} BSA-phthalate and eluted with 50 mm NaCl.

screened for antibody production using both the CEA and BSA-phthalate target plates. A second screen produced 20 wells with dual activity (Table). Positive wells were subcloned on an irradiated feeder layer (HT1080). The supernatants from cultures with dual activity were combined and concentrated by ammonium salt precipitation. After overnight dialysis, the supernatant was eluted from a DEAE column using an NaCl gradient. Three pools could be identified using ELISA data (Figure 2). The majority of anti-CEA was obtained in the first pool, bispecific activity in the second pool, and anti-phthalate in the third pool. The second pool was concentrated and eluted from DEAE column at 50 mm NaCl. Fractions were incubated on CEA target plate followed by radiolabeled I^{125} BSA-phthalate. Bispecific antibodies were identified using the radioimmunoassay (Figure 3) thus confirming a single bispecific monoclonal. As expected, maximum binding was obtained with the bivalent IgB6 monoclonal (Figure 4). The two subclones (6F7 and 6F7-1) maintained antibody production over 2 months and were grown in ascites to obtain sufficient quantities for biodistribution studies.

TABLE. NUMBER OF WELLS PRODUCING ANTIBODIES IN ELISA AT 2 AND 4 WEEKS

Screen	Anti-Phthalate	Anti-CEA	Dual
2 weeks	30	111	17
4 weeks	39	60	20

DISCUSSION

Hybrid-hybridomas produce bispecific monoclonals that may be used for immunoassays or targeted therapy.[17] They can be tailored to essentially any two antigens using standard hybridoma fusion techniques. A major requirement involves the selection of parental mutation, usually by culturing in media supplemented with 8-azoquanine, neomycin, or ouabain. Other methods such as viral transection or fluorescence activated cell sorting have been described with some success.

In this study, an HAT sensitive anti-CEA hybridoma was produced by growth in 8-azoquanine and then fused with spleen cells from hapten immunized mice. The resultant hybrid-hybridoma produced antibodies, but only a small fraction represented the desired heavy and light chain recombinants. Ten recombinants are possible between the parental heavy (H1 and H2) and light (L1 and L2) chains but only the L1H1, H2L2 recombinant is bispecific. The degree of recombination is not a random event and depends on such factors as parental isotypes and the intracellular rates of heavy and light chain production.[13] Sensitive methods are required

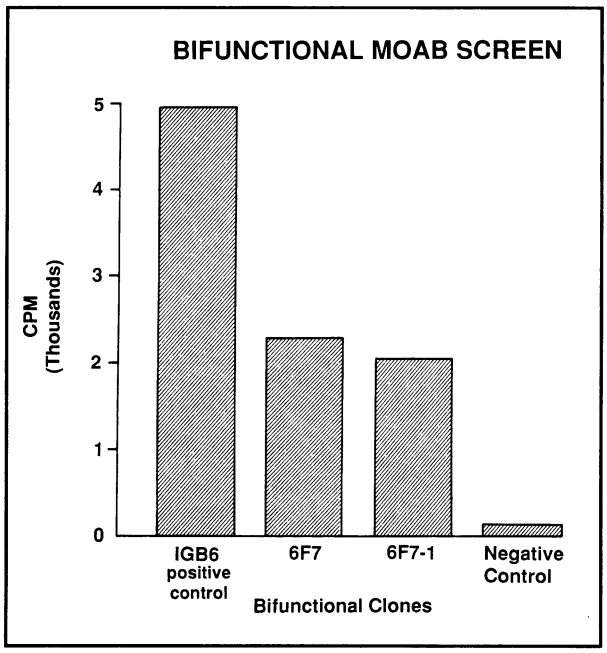

Figure 4. Bifunctional MOAB screen. Maximum binding was obtained with bivalent IgB6.

for detection because of the small amounts produced; both ELISA and radioimmunoassay were used to identify the bispecific monoclonal fraction.

As delivery agents, bifunctional antibodies were selective in their distribution and systemic exposure to isotopes, drugs, or toxins can be limited to the time of maximal antibody accumulation within a tumor. In addition, a bifunctional antibody with both tumor and hapten-binding sites allows for the colocalization or multiple haptenated reagents without needing to produce an individual hybrid-hybridoma for each therapeutic agent.

The potential use of this bispecific antibody in targeting hapten-labeled liposomes to CEA-expressing xenografts in nude mice is being investigated.

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