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Short Communication

Monoclonal and Polyclonal Antibodies Against Human Ferritin, a Nonspecific Tumor Marker

SHU-FEN CHOU[1,2] and CHIEN-YUAN CHEN[1]

ABSTRACT

The aim of this study was to produce monoclonal and polyclonal antibodies against a nonspecific tumor marker, human ferritin. Hyperimmune ICR mice produced polyclonal antibodies after injection with 0.5 mL pristane, and were injected with NS-1 myeloma cells 2 weeks later. Hyperimmune Balb/c mice were used for the production of monoclonal antibodies (MAbs). Mice were immunized four times, given a final boost, and their spleen cells were collected and fused with NS-1 myeloma cells under the presence of PEG 1500. The fused cells were then selected in the HAT-RPMIX medium. Anti-ferritin antibody-secreting hybridoma cell lines with high titer were cloned by enzyme-linked immunoadsorbent assay (ELISA) and then subcloned by limiting dilution in 15% fetal bovine serum (FBS) HT-RPMIX medium. Five murine hybridoma-producing anti-ferritin MAbs were obtained and designated 1AD11F9, 1AD11E11, 2AD11D2, 2AD11A5, and 3AD11G8. Isotypes of these MAbs were identified as IgM heavy chain and κ light chain. Hitrap Protein A and Hitrap IgM purification column were used for the purification of polyclonal and monoclonal antibodies, respectively.

INTRODUCTION

Ferritins are a class of iron storage protein widely distributed in vertebrates, invertebrates, plants, fungi, and bacteria.[1,2] In addition to iron storage, ferritin acts as a cytoprotective antioxidant (iron sequestrant) in pancreatic β-cells and endothelial cells, regulates the expression of globin genes in erythroid cells, and represses mRNA translation and proliferation of myeloid progenitor cells.[3]

Ferritin has generally been thought to function as a "house keeper" storage protein that can release iron required for cellular proliferation (e.g., ribonucleotide reductase) and metabolic renewal (e.g., cytochrome synthesis). Ferritin may also play a protective role against toxic effects of iron overload in cells.[3] Elevated levels of available iron can also promote growth of tumors[4] and infectious microorganisms.[5] At extremely elevated iron levels, ferritin can be disproportionately increased,[6] however, an increased level of ferritin is known as a nonspecific marker of inflammatory processes and neoplasms. Sera of patients with a variety of tumors (e.g., human breast cancer,[7] renal cell carcinoma,[8,9] hepatocellular carcinoma,[10,11] larynx cancer and malignant neoplasms of maxill[12] etc.) or significant disease (e.g., HIV infection, Still's disease, leukocytosis, reactive hemophagocytic syndrome[13] etc) are known to have an elevated ferritin level.

In this study, we produced anti-ferritin antibodies including polyclonal and monoclonal antibodies for application in the development of tumor marker immunosensors.

MATERIALS AND METHODS

The NS-1 myeloma cell line was a gift from Dr. Rong Huay Juang in the Agriculture Chemistry Department of Taiwan University, Taiwan, R.O.C. Balb/c mice and ICR mice (6- to 8-week-old, male) were obtained from the Experimental Animal Center of the Medical College of National Taiwan University, R.O.C.

[1]Department of Agricultural Chemistry, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 106, Taiwan, R.O.C.
[2]Department of Food Health, Chia-Nan University of Pharmacy and Science, Tainan, Taiwan, R.O.C.

Balb/c and ICR mice were given an initial intraperitoneal (i.p.) immunization with 50-μg purified antigen in complete Freund's adjuvant (Gibco, Grand Island, NY) and then boosted the antigen in incomplete Freund's adjuvant (Gibco) at 3-week intervals. After 3 months, the ICR mice produced ascites and the spleens from the Balb/c mice were used in the production of hybridoma cells.[14,15]

The hyperimmunized ICR mice were injected with 0.5 mL pristane (2,6,10,14-tetramethyldecanoic acid). Two weeks later, mice were injected with 10^6 NS-1 cells. The fluid was tapped when the mice were noticeably enlarged, but before the mice had difficulty moving. After centrifugation at 3000 $\times$ g for 10 min, supernatant was removed and the oil layer discarded.[15]

Five days before fusion and at least 3 weeks after the previous infection, a hyperimmunized mouse was given a final boost of 50 μg antigen in phosphate-buffered saline (PBS) (pH 7.0). The spleen was then removed and spleen cells (10^8) fused with NS-1 myeloma cells (10^7) using PEG 1500. Fused cells were selected in hypoxanthine, aminopterine, and thymidine (HAT)-RPMIX medium. Anti-ferritin antibody-secreting hybridoma cell lines with high titer were cloned by ELISA and then subcloned by limiting dilution in 15% fetal bovine serum (FBS) HT-RPMIX medium.[14,15]

The production of MAbs was scaled up by tissue culture in flasks and ascitic fluid in mice.

Cultures were allowed to grow until the hybridomas died, and tissue culture supernatants were collected. Debris was removed by centrifugation (1000 $\times$ g, 10 min) and supernatants were decanted from the cell pellet.[14,15] Supernatant titer was determined by ELISA.

Prime Balb/c mice were injected i.p. with 0.5 mL pristane or incomplete Freund's adjuvant. After 7–14 days, mice were injected i.p. with 5×10^5–5×10^6 hybridoma cells in 0.5 mL PBS (5 mM phosphate buffer, 0.15 M NaCl, pH 7.0). Ascitic fluid began to build up within 1–2 weeks and was tapped when the mouse was noticeably enlarged, but before the mouse had difficulty moving. The fluid was incubated at 37°C for 1 h and maintained at 4°C overnight. After centrifugation at 3000 $\times$ g, 10 min, supernatant was removed and the oil layer discarded.[14,15]

Fifty micrograms per milliliter of antigen (human ferritin) was adsorbed into a 96-well microtiter plate at 4°C overnight. After coating, the plate was washed twice with PBS. 0.2 mL gelatin-NET solution (gelatin 0.5%, NaCl 0.15 M, EDTA $\cdot$ 2 Na 5 mM. Tween 20 0.05%, Tris base 50 mM, pH 8.0) was added for blocking at room temperature. After 1 h, the plate was washed twice with PBST ($NaH_2PO_4 \cdot 2H_2O$ 10 mM, NaCl 0.13 M, Tween 20 0.05%, pH 7.0). 0.1 mL antibody solution was added to the wells and incubated at 37°C for 30 min, then 4°C for 30 min. After antibody–antigen reaction, the plate was washed three times with PBST and 0.1 mL peroxidase conjugated goat anti-mouse antibody was added to the wells and incubated at 37°C for 30 min, then at 4°C for 30 min. After 1 h of incubation, the plate was washed three times with PBST and the enzyme substrate, H_2O_2 and ABTS was added. Absorbance of the colored reaction product at 405 nm was measured by an automated ELISA reader (MR5000, Dynatech).[14,15]

Monoclonal antibodies characterization

Monoclonal cell culture supernatant (0.1 mL) was added to the ELISA plate, which had adsorbed the antigen. After 1-h incubation at room temperature, the plate was washed three times with PBST. Eight kinds of isotype goat anti-mouse Ig-peroxidase conjugates were then added to the plate for 1 h of incubation. The plate was washed three times with PBST and absorbance at 405 nm was measured.

Purification of polyclonal antibodies using Hitrap protein A column

The sample was pretreated with ammonium sulfate precipitation. The Hitrap protein A column was equilibrated with at

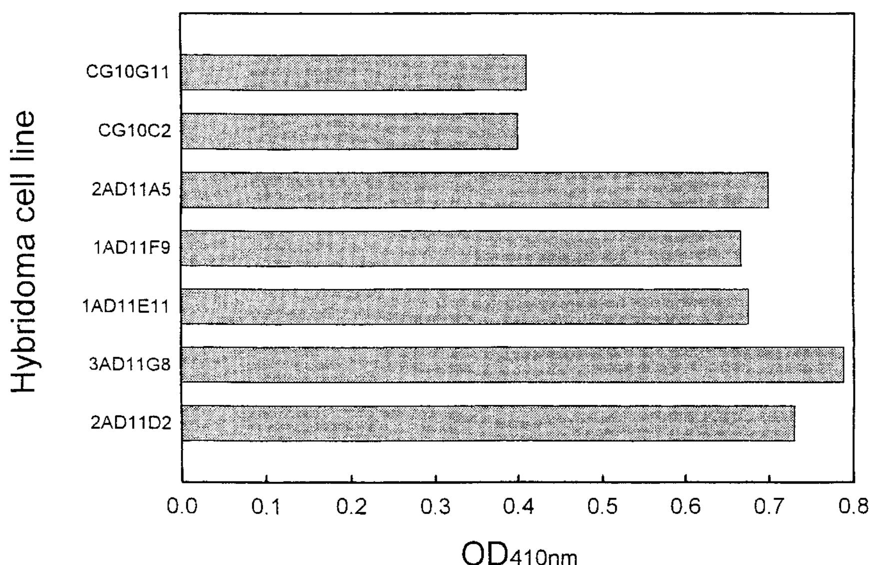

FIG. 1. Selection for anti-human ferritin MAb-secreting hybridoma cell lines with high titer.

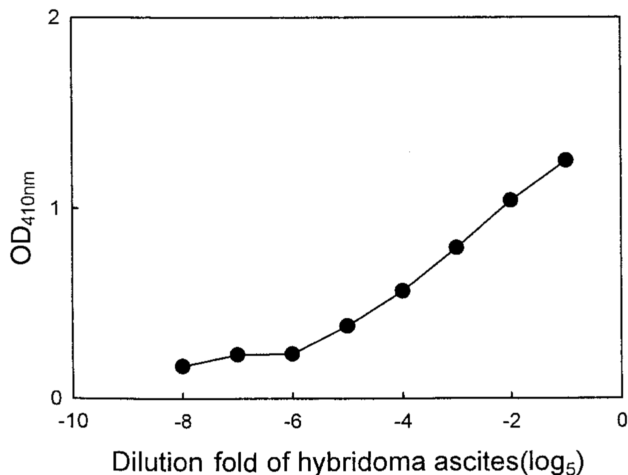

FIG. 2. Titer curve of anti-human ferritin ascites produced by i.p. injection of mice with hybridoma cells 1AD11E11. The Balb/c mice were injected with 0.5 mL pristane. After 7–14 days, the mice were injected i.p. with 10^5–10^6 hybridoma cells in 0.5 mL PBS. The ascitic fluid built up within 1–2 weeks following the injection of the cells.

least two column volumes of starting buffer (20 mM sodium phosphate, pH 7.0). It was then applied to the sample by pumping it into the column which was washed with the starting buffer for 10 column volumes or until no material appeared in the effluent and eluted with elution buffer (0.1 M citric acid buffer, pH 5.0) 1–3 column volumes. The purified IgG fraction was desalted by dialysis. Flow rates of washing and equilibration were 4 mL/min, and rates of sample application and elution were 2 mL/min.

Purification of MAbs using Hitrap IgM column

The sample was pretreated by ammonium sulfate precipitation. The Hitrap IgM column was washed with at least five column volumes of binding buffer (20 mM sodium phosphate, 0.8 M ammonium sulphate, pH 7.5), elution buffer (20 mM sodium phosphate, pH 7.5) and regeneration buffer (20 mM sodium phosphate, pH 7.5 with 30% isopropanol). The column was then equilibrated with five column volumes of the binding buffer and the sample was applied by pumping it into the column. Unbound materials were washed out with at least 15 column volumes of binding buffer. The IgM was eluted with 12 column volumes of elution buffer the column was regenerated with 7 column volumes of regeneration buffer. Flow rates of washing and equilibration were 4 mL/min, and rates of sample application and elution were 1 mL/min.

RESULTS

Ascites formation was induced in hyperimmune ICR mice (serum titer 1:6000) when injected with pristane and then NS-1 myeloma cells after two weeks. The highest dilution fold of the ascites determined by ELISA was 3125-fold. The ascites were purified using Hitrap protein A column. A single peak of protein fraction ($OD_{280\ nm}$ = 2.8) was obtained when elution buffer (Buffer B) was applied to the column (data not shown).

The five high-titer MAb-producing hybridoma cell lines selected and designated 2AD11A5, 1AD11F9, 1AD11E11, 3AD11G8, and 2AD11D2 are shown in Fig. 1. The isotypes of MAbs secreted by the five hybridoma cell lines were classified as IgM heavy chain and κ light chain using mouse-hybridoma subtyping kit. When high-titer hybridoma was injected i.p. into mice, a tumor formed locally or antibody-rich ascites developed. The titer curve of mouse anti-ferritin ascites produced with hybridoma cell line 1AD11E11 is shown in Fig. 2. The highest dilution fold of the hybridoma ascites determined by ELISA was 3125-fold.

The ascites containing monoclonal anti-ferritin antibody 1AD11F9 was purified using Hitrap protein IgM column. An affinity chromatogram is shown in Fig. 3. A single peak of protein fraction ($OD_{280\ nm}$ = 2.7) was obtained when elution buffer (Buffer B) was applied to the column.

DISCUSSION

Ferritins are a type of complete antigen that when mixed with Freund's adjuvant can stimulate a good response after injection in mice. A successful fusion procedure brings cells together with an optimal frequency of interactions between the two "parent" cell types. Unfused myeloma cells were dying out as a result of the aminopterin block. Spleen cells were dying out, with the exception of macrophages and/or fibroblasts which might be establishing themselves and beginning to divide.[14] There appeared to be a correlation between the appearance of such cells and subsequent good yield of hybrids. The cells were characteristically round with a clear membrane under phase con-

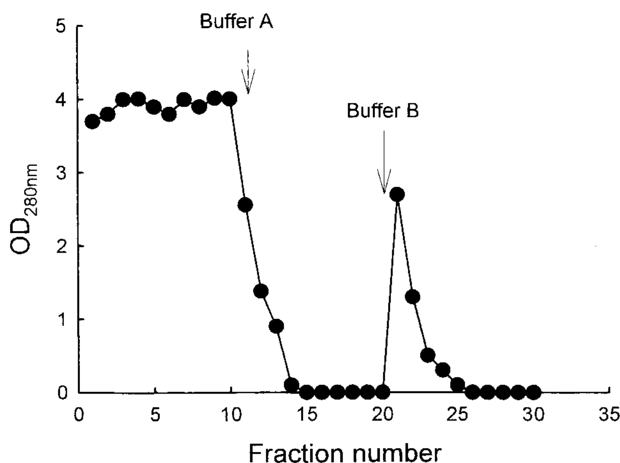

FIG. 3. Affinity chromatogram of Balb/c mice ascites producing monoclonal anti-human ferritin antibodies 1AD11F9 using Hitrap IgM purification column. 10 mL of the hybridoma ascites was applied into the column. The binding buffer (Buffer A) is a solution with pH 7.5 containing 20 mM sodium phosphate and 0.8 M ammonium sulphate. The elution buffer (Buffer B) is a 20 mM sodium phosphate with pH 7.5. Flow rates of washing and equilibration were 4 mL/min, and rates of sample application and elution were 1 mL/min with 1 mL/ fraction.

trast. When the medium in the culture turned yellow, the cultures were screened to determine antibody production and positive colonies by ELISA were selected for expansion and subcloning. Limiting dilution was performed by adding 15% FBS HT-RPMIX medium to replace the conventional method. For maintenance and expansion of MAb-producing hybridoma cell lines, class and subclass were determined on MAb prepared in culture, rather than in mice, to avoid other classes and subclasses, originating from the mouse model.

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Address reprint requests to:
Dr. Chein-Yuan Chen
Department of Agricultural Chemistry
National Taiwan University, No. 1, Sec. 4
Roosevelt Road
Taipei 106
Taiwan, R.O.C.

E-mail: chenyuan@ccms.ntu.edu.tw

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