嘉南藥理科技大學專題研究計畫成果報告

前列腺特異性抗原單株及多株抗體之生產

計畫類別：☑個別型計畫　　□整合型計畫

計畫編號：90-FH-13

執行期間：90 年 1 月 1 日至 90 年 12 月 31 日

計畫主持人：周淑芬

共同主持人：

計畫參與人員：

執行單位：食品衛生系

Production and Purification of Monoclonal and Polyclonal Antibodies Against Prostate Specific Antigen, a Prostate Cancer Serum Marker

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Abstract

The aim of this study was to produce monoclonal and polyclonal antibodies against prostate specific antigen (PSA), a prostate cancer serum marker. Hyperimmune ICR mice produced polyclonal antibodies after injection with 0.5 ml pristane, and were injected with NS-1 myeloma cells two weeks later. Hyperimmune Balb/c mice were used for the production of monoclonal antibodies (MAbs). After these mice were immunized four times and given a final boost, 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 hypoxathine, aminopterine, and thymidine (HAT)-RPMIX medium. Anti-PSA antibody-secreting hybridoma cell lines with high titer were cloned by enzyme-linked immunosorbent assay (ELISA) and then subcloned by limiting dilution in 15% fetal bovine serum (FBS) HT-RPMIX medium. Twelve murine hybridoma producing anti-PSA MAbs were obtained and designated C3m1G11, B3m1E5, C3m1E8, C3m1C5, C3m2F4, C3m1F8, C3m2B3, C3m2E6, B3m2B11, B3m2F2, C3m2C7, C3m2D9. Isotypes of these MAbs were identified as IgG2a heavy chain and κ light chain. Hitrap Protein A column was used for the purification of polyclonal and monoclonal antibodies.

1. Introduction

Prostate specific antigen (PSA) is a 32- to 33-kDa single-chain glycoprotein [1], which has been characterized as a serine protease with restricted chymotrypsin-like specificity belonging to the human kallikrein gene family [1-3]. The protein is secreted by the epithelial cells of the prostate [4], and levels increase in patients with prostate cancer (PCa) [5]. Therefore, immunoassays measuring serum PSA concentrations have been used as screening tests or to facilitate clinical management of PCa [6]. However, increased PSA concentrations in serum also occur in patients with benign prostate hyperplasia (BPH) [7-9] and this reduces the predictive value of PSA measurement for early diagnosis.

Different concepts have been used to enhance the specificity of serum PSA measurement without losing sensitivity, including PSA velocity, density and age-specific reference ranges [10-12]. Furthermore, PSA exists in serum with several forms, of which the most important are the free form of PSA (fPSA), PSA complexed to α-1-antichymotrypsin (PSA-ACT), and PSA complexed to α-2-macroglobulin. The sum of PSA-ACT and fPSA mainly accounts for the total PSA (tPSA) in the serum [13,14]. The observation of significant differences in the proportion of fPSA in patients with PCa when compared with those with BPH or in healthy men originated a new concept based on the ratio of serum fPSA to serum tPSA, the free-to-total PSA ratio (f/tPSA). Recently, a better discrimination between PCa and BPH was reported using f/tPSA rather than tPSA, within the diagnostic 'gray zone' of tPSA levels of 4-10 ng/ml [15-18]. In a screening population of men with total PSA levels in the 'gray zone' about 22% have PCa [19].

The aim of this study was to produce anti-PSA antibodies including polyclonal and monoclonal antibodies for application in the development of tumor marker immunosensors. In this study, polyclonal and monoclonal anti-PSA antibodies were produced, characterized, and purified.

2. Materials and Methods

2.1 Reagents

1. PSA from human seminal fluid (Chemicon Intl., Temecula, CA, U.S.A.) SDS-PAGE$\geqq$90% 2. RPMIX: RPMI 1640 (Seromed, Berlin, Germany) was supplemented with fetal bovine serum (FBS) (Hyclone, Logan, Utah, U.S.A.) 12%, L-glutamine (200 mM, GibcoBRL, Grand island, NY, U.S.A.) 1%, Pen-Strep (10000 U penicillin G and 10 mg streptomycin/ml solution, 100X, GibcoBRL, Grand island, NY, U.S.A.) 1%, fungizon (250 μg/ml, GibcoBRL, Grand island, NY, U.S.A.) 1% and sodium pyruvate (100 mM, GibcoBRL, Grand island, NY, U.S.A.) 1%. 3. Fruend's adjuvant (complete and incomplete, GibcoBRL, Grand island, NY, U.S.A.) 4. Peroxidase conjugated goat anti-mouse IgA, IgG, IgM (Capple, Malvern, PA, U.S.A.) 5. ABTS (2,2-azino-di-[3-ethyl-benzthiazoline sulfonate] diammonium salt) (Sigma, St. Louis, MO, U.S.A.) 6. PEG1500 (polyethylene glycol 1500) (Roche Diagnostics GmbH, Mannheim, Germany) 7.

HAT (hypoxanthine 10 mM, thymidine 1.6 mM, aminopterin 1.76 mg/100ml) (GibcoBRL, Grand island, NY, U.S.A.) 8. HT (hypoxanthine 10 mM, thymidine 1.6 mM) (GibcoBRL, Grand island, NY, U.S.A.) 9. Hitrap Protein A column (Amersham Pharmacia Biotech, Inc., Piscataway, NJ. U.S.A.)

2.2 Materials

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 (six to eight weeks old, male) were obtained from the Experimental Animal Center of the Medical College of National Taiwan University, R.O.C..

2.3 Immunization

All 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 three months, the ICR mice could produce ascites and the spleens from the Balb/c mice were used in the production of hybridoma cells [20,21].

2.4 Procedure of Enzyme-linked immunosorbent assay (ELISA)

Fifty micrograms per milliliter of antigen (human PSA) was adsorbed into a 96-well microtiter plate at 4℃ overnight. After coating, the plate was washed twice with phosphate buffered saline (PBS) (5 mM phosphate buffer, 0.15 M NaCl, pH 7.0). 0.2 ml of gelatin-NET solution (gelatin 0.5%, NaCl 0.15 M, EDTA • 2Na 5 mM. Tween 20 0.05%, Tris base 50 mM, pH 8.0) was then added to the plate for blocking at room temperature. After 1 hr, the plate was washed twice with PBST (NaH_2PO_4 • $2H_2O$ 10 mM, NaCl 0.13 M, Tween 20 0.05%, pH 7.0). 0.1 ml of antibody solution was added to the wells and incubated at 37℃ for 30 min, then 4 ℃ for 30 min. After antibody-antigen reaction, the plate was washed three times with PBST and 0.1 ml of peroxidase conjugated goat anti-mouse antibody was added to the wells and incubated at 37℃ for 30 min, then at 4℃ for 30 min. After 1 hr of incubation with these antibodies, the plate was washed three times with PBST and the enzyme substrate, H_2O_2 and ABTS was added. Absorbance at 405 nm of the colored reaction product was measured by an automated ELISA reader (MR5000, Dynatech) [20,21].

2.5 Production of Polyclonal Antibodies

The hyperimmunized ICR mice were injected with 0.5 ml pristane (2,6,10,14-tetramethyldecanoic acid). Two weeks later, the 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 x g for 10 min, supernatant was carefully removed and the oil layer discarded [21].

2.6 Production of Monoclonal Antibodies (MAbs)

2.6.1 Hybridization

Seven days before fusion, a hyperimmunized mouse was given a final boost of 50 μg antigen in PBS (pH 7.0) at least three weeks after the previous injection. 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 the hypoxathine, aminopterine, and thymidine (HAT)-RPMIX medium. Anti-PSA antibody-secreting hybridoma cell lines with high titer were cloned by ELISA and then subcloned by limiting dilution in 15% FBS HT-RPMIX medium [20,21].

2.6.2 Scale-up of MAbs Production

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

2.6.2.1 Collection of tissue culture supernatants

The cultures were allowed to grow until the hybridomas died, and the tissue culture supernatants were collected. Debris was removed by centrifugation (1000xg, 10 min) and supernatants were decanted from the cell pellet [20,21]. Supernatant titers were determined by ELISA.

2.6.2.2 Collection of ascites

Prime Balb/c mice were injected i.p. with 0.5 ml of pristane or incomplete Freund's adjuvant. After 7-14 days, the mice were injected i.p. with $5x10^5 \sim 5x10^6$ hybridoma cells in 0.5 ml PBS. 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℃ for 1 hr and maintained at 4 ℃ overnight. After centrifugation at 3000xg, 10 min, supernatant was carefully removed and the oil layer discarded [20,21].

2.7 Classification of MAbs

Monoclonal cell culture supernatant (0.1 ml) was added to the ELISA plate that had adsorbed the antigen. After 1 hr of 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 hr of

incubation. The plate was washed three times with PBST and absorbance at 405 nm was measured.

2.8 Purification of Polyclonal and Monoclonal Antibodies Using Hitrap Protein A column

The sample was pretreated by the ammonium sulfate precipitation. The Hitrap Protein A column was equilibrated with at least two column volumes of binding buffer (Buffer A) (20 mM sodium phosphate, pH 7.0). It was then applied to the sample by pumping it into the column, which was washed with Buffer A for 10 column volumes or until no material appeared in the effluent. It was eluted with elution buffer (Buffer B) (0.1 M citric acid buffer, pH 5.0) 1-3 column volumes. The purified IgG fraction could be desalted by dialysis. Flow rates of washing and equilibration were 4 ml/min, and rates of sample application and elution were 2 ml/min.

3. Results

3.1 Production and Purification of Polyclonal Antibodies

Ascites formation could be 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_{280nm}=3.8) was obtained when Buffer B was applied to the column (data not shown).

3.2 Production and Classification of MAbs

In this experiment, the twelve high-titer MAbs-producing hybridoma cell lines selected and designated C3m1G11, B3m1E5, C3m1E8, C3m1C5, C3m2F4, C3m1F8, C3m2B3, C3m2E6, B3m2B11, B3m2F2, C3m2C7, C3m2D9 are shown in Fig. 1. The isotypes of MAbs secreted by the twelve hybridoma cell lines were classified as IgG2a 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-PSA ascites produced with hybridoma cell line C3m2F4 is shown in Fig. 2. The highest dilution fold of the hybridoma ascites determined by ELISA was 3125-fold.

3.3 Purification of MAbs

The ascites containing anti-PSA MAbs C3m2F4 was purified using Hitrap Protein A column. An affinity chromatogram of anti-PSA MAbs C3m2F4 from Balb/c mice ascites using Hitrap A purification column is shown in Fig. 3. A single peak of protein fraction (OD_{280nm}=3.3) was obtained when elution buffer (Buffer B) was applied to the column.

4. Discussion

PSA are a type of complete antigens. This antigen mixed with Freund's adjuvant can stimulate a good response when injected into mice. A successful fusion procedure could bring 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 [20]. It appeared to be a correlation between the appearance of such cells and subsequent good yields of hybrids. The cells were characteristically round with a clear membrane under phase contrast. 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. In this study, limiting dilution was performed by adding 15% FBS HT-RPMIX medium to replace the conventional method that used feeder cells applied for hybridoma cells in the 96-well microtiter plate. Maintenance and expansion of MAbs- producing hybridoma cell lines were important. The class and subclass should be 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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Figure legends:

Fig.1. Selection for anti-human PSA MAb-secreting hybridoma cell lines with high titer

Fig.2. Titer curve of anti-human PSA ascites produced by i.p. injection of mice with hybridoma cells C3m2F4. The Balb/c mice were injected 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.

Fig.3. Affinity chromatogram of Balb/c mice ascites producing monoclonal anti-human PSA antibodies C3m2F4 using Hitrap protein A purification column. 10ml of the hybridoma ascites was applied into the column. The binding buffer (Buffer A) is a solution containing 0.05 M Tris-HCl, 3 M NaCl (pH 7.8). The elution buffer (Buffer B) is a 0.1 M citrate buffer (pH 5.0). Flow rate of washing and equilibration is 4

ml/min. Flow rate of sample application and elution is 1 ml/min (1 ml/fraction).