嘉南藥理科技大學專題研究計畫成果報告

C 反應蛋白單株及多株抗體之生產

計畫類別：V 個別型計畫 □整合型計畫

計畫編號：CNBT93-14

執行期間：93 年 1 月 1 日至 93 年 12 月 31 日

計畫主持人：周淑芬

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執行單位：生物科技系

中華民國 94 年 2 月 1 日
Production and Purification of New Monoclonal and Polyclonal Antibodies Against C-reactive Protein (CRP)

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Abstract

The aim of this study was to produce monoclonal and polyclonal antibodies against C-reactive protein (CRP). 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 hypoxanthine, aminopterin, and thymidine (HAT)-RPMIX medium. Anti-CRP 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. Ten murine hybridoma producing anti-CRP MAbs were obtained and designated CRP-2E, CRP-2B, CRP-5E, CRP-11H, CRP-11F, CRP-12C, CRP-3C, CRP-12F, CRP-11G, CRP-9G. Aside from the isotypes of CRP-3C, CRP-12C and CRP-5E were identified as IgG2b heavy chain and \( \kappa \) light chain, those of the others were 6. PEG1500 (polyethylene glycol 1500) (Roche Diagnostics GmbH, Mannheim, Germany) 7. chain. Hitrap rProtein A column was used for HAT (hypoxyantine 10 mM, thymidine 1.6 mM, the purification of polyclonal and monoclonal aminoprotein) 1.76 mg/100mL) (GibcoBRL, Grand island, NY, U.S.A.) 8. HT (hypoxyantine 10 mM, thymidine 1.6 mM) (GibcoBRL, Grand island, NY, U.S.A.) 9. Hitrap rProtein A column (Amersham U.S.A.)

1. Introduction

The C-reactive protein (CRP) is synthesized by the liver in response to interleukin-6 and well known as one of classical acute-phase reactants. The NS-1 myeloma cell line was a gift from Dr. Rong Huay Juang in the Agriculture Chemistry Department of Chia-Nan University of Pharmacy and Science. However, the CRP level may range from a normal level <5 to 500 mg/L during the body’s general, non-specific Balb/c mice and ICR mice (six to eight weeks old, response to infectious and measurement of CRP male) were obtained from the Experimental Animal Center of the Medical College of National Taiwan University, R.O.C. processes, such as rheumatoid arthritis. It has recently been suggested that a marker of inflammation, along with serum cholesterol, may be critical component in the development and progression of atherosclerosis. Since the early 1990s, a growing body of evidence has supported the idea that cardiovascular diseases, including coronary heart disease, ischemic stroke, and acute peripheral vascular disease, develop, at least in part, because of a chronic, low-level CRP of the vascular endothelium. However, the prospect of using CRP as a predictor of future vascular risks faces a big obstacle because existing assay methods.

2. Materials and Methods

2.1 Reagents

1. CRP from human plasma (Sigma Chem. Co., St. Louis, MO, U.S.A) 2. RPMIX: RPMI 1640 supplemented with fetal bovine serum (FBS) (Hyclone, Logan, UT, U.S.A.) 12%, L-glutamine (200 mM, GibcoBRL, Grand island, NY, U.S.A.) 1%, aminopterin, and thymidine (HAT)-RPMIX Pen-Strep (10000 U penicillin G and 10 mg medium. Anti-CRP antibody-secreting streptomycin/mL solution, 100X, GibcoBRL, hybridoma cell lines with high titer were cloned (complete and incomplete, GibcoBRL, MAbs were obtained and designated CRP-2E, Grand island, NY, U.S.A.) 4. Peroxidase CRP-2B, CRP-5E, CRP-11H, CRP-11F, conjugated goat anti-mouse IgA, IgG, IgM CRP-12C, CRP-3C, CRP-12F, CRP-11G, (Capple, Malvern, PA, U.S.A.) 5. ABTS (2, 2-azino-di- [3-ethyl-benzthiazoline sulfonate] 10 mM, thymidine 1.6 mM, the purification of polyclonal and monoclonal aminoprotein 1.76 mg/100mL) (GibcoBRL, Grand island, NY, U.S.A.) 8. HT (hypoxyantine 10 mM, thymidine 1.6 mM) (GibcoBRL, Grand island, NY, U.S.A.) 9. Hitrap rProtein A column (Amersham U.S.A.)
intraperitoneal (i.p.) immunization with 10 μg by limiting dilution in 15% FBS HT-RPMIX purified antigen in complete Freund’s adjuvant medium (Gibco, Grand Island, NY) and then boosted the 2.6.2 Scale-up of MAbs Production antigen in incomplete Freund’s adjuvant (Gibco) The production of MAbs was scaled up by at 3-week intervals. After three months, the ICR tissue culture in flasks and ascitic fluid in mice. mice could produce ascites and the spleens from 2.6.2.1 Collection of tissue culture supernatants the Balb/c mice were used in the production of The cultures were allowed to grow until the hybridoma cells (Gibco). 2.4 Procedure of Enzyme-linked immunosorbent assay (ELISA) Fifty micrograms per milliliter of antigen (CRP) and supernatants were decanted from the cell was adsorbed into a 96-well microtiter plate at 4°C overnight. After coating, the plate was by ELISA, washed twice with phosphate buffered saline 2.6.2.2 Collection of ascites (PBS) (5 mM phosphate buffer, 0.15 M NaCl, pH 7.0). 0.2 ml of gelatin-NET solution (gelatin 0.5%, mL of pristane or incomplete Freund’s adjuvant, NaCl 0.15 M, EDTA 2Na 5 mM, Tween 20) After 7-14 days, the mice were injected i.p. with 0.05%, Tris base 50 mM, pH 8.0) was then 5 × 10^5–5 × 10^6 hybridoma cells in 0.5 mL added to the plate for blocking at room 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. After 1 hr, the plate was washed weeks and was tapped when the mouse was twice with PBST (NaH2PO4 · 2H2O 10 mM, NaCl noticeably enlarged, but before the mouse had 0.13 M, Tween 20 0.05%, pH 7.0). 0.1 mL of difficulty moving. The fluid was incubated at 37°C antibody solution was added to the wells and for 1 hr and maintained at 4°C overnight. After incubated at 37°C for 30 min, then 4°C for 30 centrifugation at 3000 × g, 10 min, supernatant min. After antibody-antigen reaction, the plate was carefully removed and the oil layer was washed three times with PBST and 0.1 mL discarded (Gibco). 2.7 Classification of MAbs of peroxidase conjugated goat anti-mouse antibody was added to the wells and incubated Monoclonal cell culture supernatant (0.1 mL) at 37°C for 30 min, then at 4°C for 30 min. After was added to the ELISA plate that had adsorbed 1 hr of incubation with these antibodies, the plate the antibody. After 1 hr of incubation at room washed three times with PBST and the temperature, the plate was washed three times enzymatic substrate, H2O2 and ABTS was added. with PBST. Eight kinds of isotype goat Absorbance at 405 nm of the colored reaction anti-mouse IgG-peroxidase conjugates were then product was measured by an automated ELISA added to the plate for 1 hr of incubation. The plate was washed three times with PBST and absorbance at 405 nm was measured. 2.5 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 sample was pretreated by the The fluid was tapped when the mice were ammonium sulfate precipitation. The Hitrap noticeably enlarged, but before the mouse had rProtein A column was equilibrated with at least difficulty moving. After centrifugation at 3000×g for 10 min, supernatant was carefully removed and the oil layer discarded. 2.6 Production of Monoclonal Antibodies (MAbs) 2.6.1 Hybridization Seven days before fusion, a hyperimmunized mouse was given a final boost of 10 μg antigen column volumes. The purified IgG fraction could in PBS (pH 7.0) at least three weeks after the be desalted by dialysis. Flow rates of washing previous injection. The spleen was then removed and equilibration were 4 mL/min, and rates of and spleen cells (10^3) fused with NS-1 myeloma sample application and elution were 2 mL/min. cells (10^3) using PEG 1500. Fused cells were 3. Results selected in the hypoxathine, aminopterine, and thymidine (HAT)-RPMIX medium. Anti-CRP Antibodies antibody-secreting hybridoma cell lines with high Ascites formation could be induced in titer were cloned by ELISA and then subcloned hyperimmune ICR mice (serum titer 1:10^6) when
injected with pristane and then NS-1 myeloma should be determined on MAb prepared in
cells after two weeks. The highest dilution fold of culture, rather than in mice, to avoid other
the ascites determined by ELISA was 15625-fold. classes and subclasses, originating from the
The ascites were purified using Hitrap r Protein A mouse model.
column. A single peak of protein fraction References
(OD<sub>280nm</sub>=3.8) was obtained when Buffer B was 1.
applied to the column (data not shown).

### 3.2 Production and Classification of MAbs

In this experiment, the ten high-titer MAbs-producing hybridoma cell lines selected
and designated are shown in Fig. 1. The
isotypes of MAbs secreted by the seven
hybridoma cell lines were classified as IgG2b heavy chain and κ light chain using
mouse-hybridoma subtyping kit. When high-titer 3.
hybridoma was injected i.p. into mice, a tumor
formed locally or antibody-rich ascites developed.
The titer curve of mouse anti-CRP ascites
produced with hybridoma cell line CRP-9G is
shown in Fig. 2. The highest dilution fold of the 4.
hybridoma ascites determined by ELISA was
15625-fold.

### 3.3 Purification of MAbs

The ascites containing anti-CRP MAbs
CRP-9G was purified using Hitrap r Protein A 5.
column. An affinity chromatogram of anti-CRP MAbs CRP-9G from Balb/c mice ascites using
Hitrap r Protein A purification column is shown in
Fig. 3. A single peak of protein fraction
(OD<sub>280nm</sub>=3.7) was obtained when elution buffer 6.
(Buffer B) was applied to the column.

### 4. Discussion

CRP is a kind of complete antigens. This
antigen mixed with Freund's adjuvant can stimulate a good response when injected into 7.
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 8.
dying out, with the exception of macrophages and/or fibroblasts, which might be establishing themselves, and beginning to divide (9). It appeared to be a correlation between the appearance of such cells and subsequent good 9.
yields of hybrids. The cells were characteristically round with a clear membrane under phase contrast. When the medium in the 10.
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 Figure legends:

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

Fig.2. Titer curve of anti- CRP ascites
produced by i.p. injection of mice with

References

hybridoma cells CRP-9G. 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-CRP antibodies CRP-9G using Hitrap Protein A purification column. 10 mL 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).
Fig. 1