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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 hypoxathine, aminopterine, 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 IgA and κ light chain, those of the others were identified as IgG2b heavy chain and κ light chain. Hitrap rProtein A column was used for the purification of polyclonal and monoclonal antibodies.

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 and as a marker of inflammation. The serum CRP level may rise from a normal level <5 to 500 mg/L during the body's general, non-specific response to infectious and measurement of CRP concentration has been used as a clinical tool for monitoring autoimmune disease and infectious 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 [1,2]. Since the early 1990s, a growing body of evidence has supported the idea that cardiovascular dieases, including coronary heart disease, ischemic stroke, and acute myocardial infraction, as well as peripheral vascular disease, develop, at least in part, because of a chronic, low-level CRP of the vascular endothelium[3-8]. However, the prospect of using CRP as a predictor of future vascular risks faced a big obstacle because existing assay methods.

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

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 (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 μ g/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 rProtein 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 10 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 [9,10].

2.4 Procedure of Enzyme-linked immunosorbent assay (ELISA)

Fifty micrograms per milliliter of antigen (CRP) 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) [9,10].

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×g for 10 min, supernatant was carefully removed and the oil layer discarded [9].

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 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-CRP antibody-secreting hybridoma cell lines with high titer were cloned by ELISA and then subcloned by limiting dilution in 15% FBS HT-RPMIX medium [9,10].

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 (1000 × g, 10 min) and supernatants were decanted from the cell pellet [9,10]. 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 $5 \times 10^5 \sim 5 \times 10^6$ 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 3000 × g, 10 min, supernatant was carefully removed and the oil layer discarded [9,10].

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 rProtein A column

The sample was pretreated by the ammonium sulfate precipitation. The Hitrap rProtein 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 1:10^4) when

injected with pristane and then NS-1 myeloma cells after two weeks. The highest dilution fold of the ascites determined by ELISA was 15625-fold. The ascites were purified using Hitrap rProtein 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 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 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 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 rProtein A column. An affinity chromatogram of anti-CRP MAbs CRP-9G from Balb/c mice ascites using Hitrap rProtein A purification column is shown in Fig. 3. A single peak of protein fraction (OD_{280nm}=3.7) was obtained when elution buffer (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 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 [(9)]. 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.

References

1. Whicher, J., Biasucci, L., Rifai, N., Inflammation, the acute phase response and atherosclerosis, Clin. Chem. Lab. Med., 1999; 37: 495-503.
2. Pentikanen, M. O., Oorni, K., Ala-Korpela, M., Kovanen, P. T., Modified LDL-trigger of atherosclerosis and inflammation in the arterial intima, J. Intern. Med., 2000; 247: 359-370.
3. Ridker, P. M., Cushman, M., Stampfer, M. J., Tracy, R. P., Hennekens, C. H., Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men, N. Engl. J. Med., 1997; 336: 973-979.
4. Ridker, P. M., Cushman, M., Stampfer, M. J., Tracy, R. P., Hennekens, C. H., Plasma concentration C-reactive protein and risk of developing peripheral vascular disease, Circulation, 1998; 97: 425-428.
5. Koenig, W., Sund, M., Frohlich, M., et al. C-reactive protein, a sensitive marker of inflammation, predicts future risk of coronary heart disease in initially healthy middle-aged men, Circulation, 1999; 99: 237-242.
6. Haverkate, F., Thompson, S. G., Pyke, S. D. M., Gallimore, J. R., Pepys, M. B., Production of C-reactive protein and risk of coronary events in stable and unstable angina, Lancet, 1997; 349: 462-466.
7. Biasucci, L. M., Liuzzo, G., Grillo, R. L., et al. Elevated level of C-reactive protein at discharge in patients with unstable angina predict recurrent instability, Circulation, 1999; 99: 855-860.
8. de Winter, R. J., Bholasingh, R., Lijmer, J. G., et al. C-reactive protein and troponin I in patients with unstable angina or non-Q-wave myocardial infarction, Cardiovasc. Res., 1999; 42: 240-245.
9. Hurrell, J. G. R., Monoclonal hybridoma antibodies: Techniques and Application, CRC press, Inc., Florida, U.S.A.,1982.
10. Chuang, Z. H., Studies on sucrose synthetase from rice, Doctor Thesis of the Graduated Institute of Agriculture Chemistry in National Taiwan University, Taipei, Taiwan, 1985.

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

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 rProtein 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).

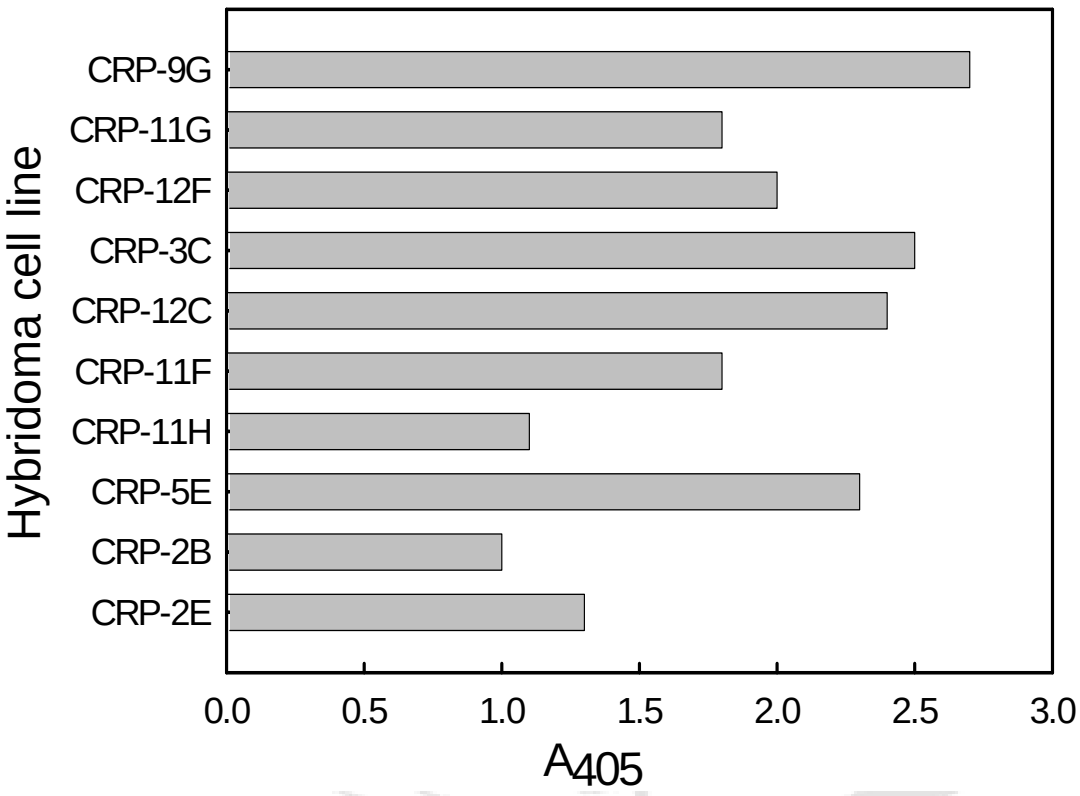
Hybridoma cell line
CRP-9G
CRP-11G
CRP-12F
CRP-3C
CRP-12C
CRP-11F
CRP-11H
CRP-5E
CRP-2B
CRP-2E
0.0
0.5
1.0
1.5
2.0
2.5
3.0
A405

Fig.1

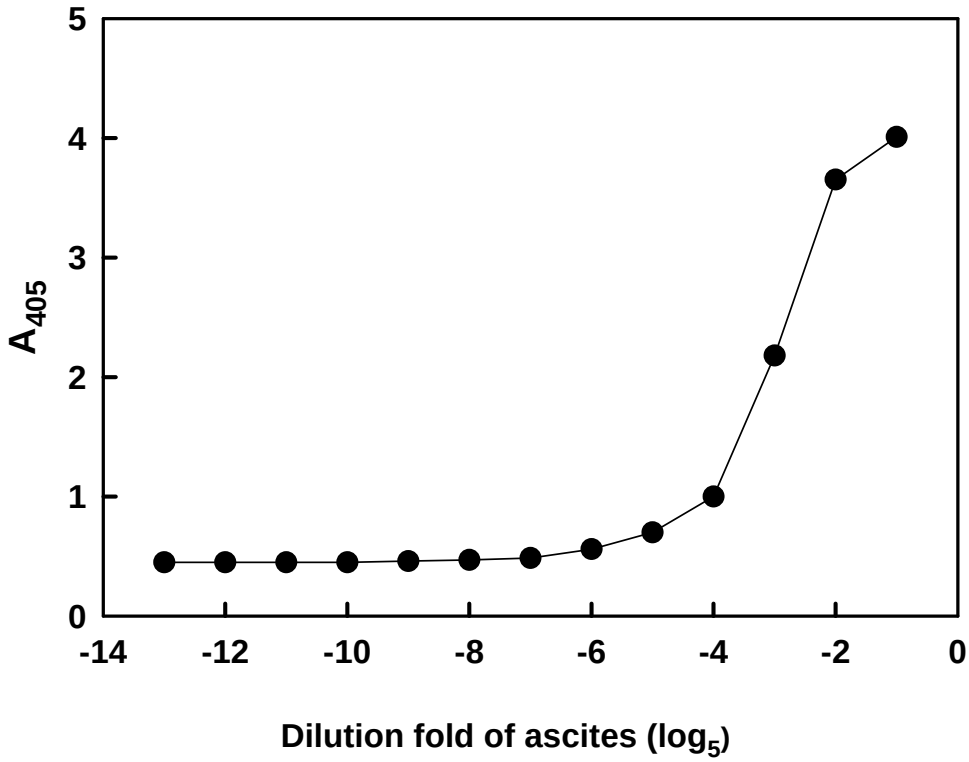
5
4
3
2
1
0
A405
-14
-12
-10
-8
-6
-4
-2
0
Dilution fold of ascites (log5)

Fig.2

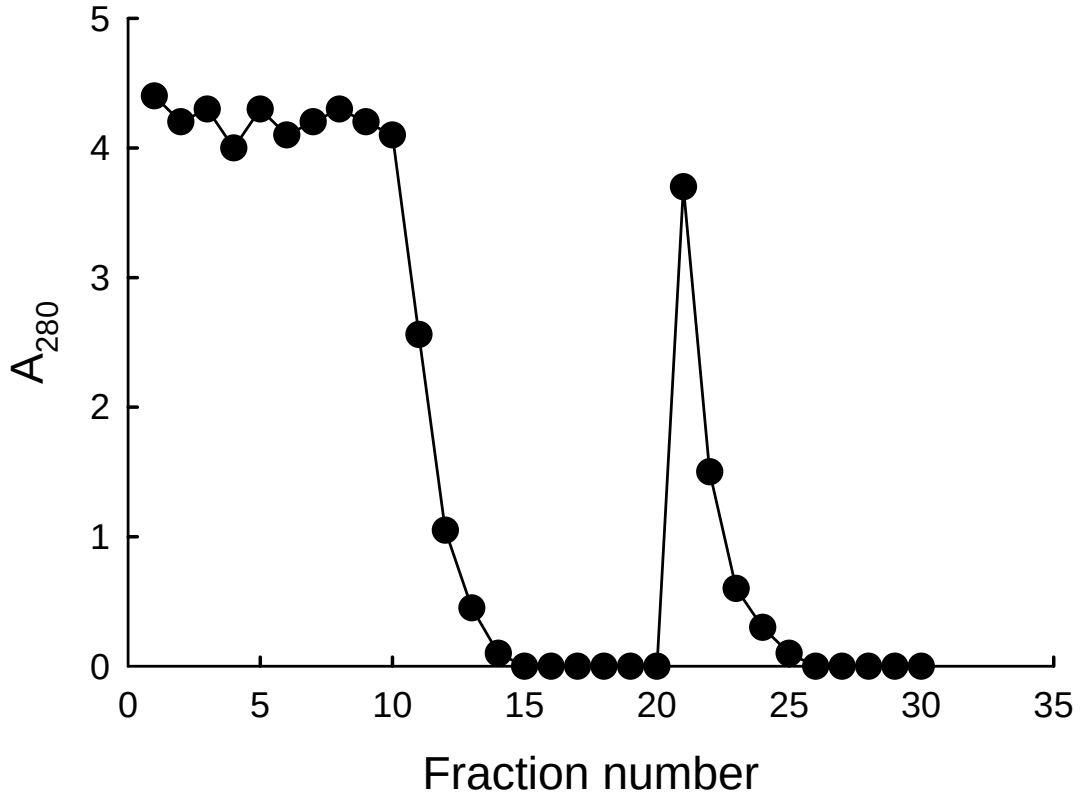
5
4
3
2
1
0
A280
0
5
10
15
20
25
30
35
Fraction number

Fig.3