PURIFICATION OF MONOCLONAL ANTIBODY 3H11 AGAINST GASTRIC CANCER FOR *IN VIVO* USE

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Monoclonal antibody (McAb) 3H11 against gastric cancer was grown in the mouse ascites system. To acquire a clinical grade product for cancer radioimmuno-imaging was purified by two step high performance liquid chromatography (HPLC) protocol using protein A and high-performance hydroxylapatite (HPHT). An analysis of data reported shows the two step HPLC method to be the best purification procedure. This protocol satisfies purity and immunoreactivity requirement, and provides an sample sterility, free-pyrogens, free-mycoplasma and non-specific IgG contamination. This procedure described was capable of generating large amounts of clinical grade monoclonal antibody.

Key words: Monoclonal antibody, Protein A, Hydroxylapatite, HPLC, Purification.

Since the first description of McAb technology by Kohler and Milstein in 1975, many immunoglobulin purification methods have been reported in the literature.[1-3] During recent years, interest has increased in this field because of the extensive application of McAbs to radioimmuno-imaging and immunotherapy of human tumor lesions. Investigators have focused on developing purification methods which provide sample suitable for clinical application. Despite all studies so far reported the methods currently in use are still based on conventional protein purification techniques. Antibodies may be separated according to charge or size, and maybe purified by affinity chromatography using column with either protein A, protein G, anti-mouse immunoglobulins or specific antigen immobilized on to the solid phase support.

The aim of this work was to develop a purification method for a murine McAbs. The principle of this method should be generally applicable for other monoclonal IgG preparation and the final product should meet the requirements of GMP concerning McAbs for therapeutic purposes. The purification process involves a combination of protein A and HPHT HPLC. It provides highly purified McAb 3H11 product, furthermore the McAb purification system for automated.

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MATERIAL AND METHODS

Monoclonal Antibody

IgG2b McAb against gastric cancer was produced by hybridoma 3H11,[4] grown in the mouse ascites system. About 100 ml of ascites fluids were collected from Balb/c mice and pooled to obtain a single batch. An aliquot of the batch was submitted to National Institute for the Control of Pharmaceutical and Biological Products for a series of control according to requirements for the production and quality control of murine origin intended for use in humans. Absence of mycoplasma, as well as of murine viruses and human retoviruses was investigated and confirmed by appropriate techniques.

Purification of McAb

HPLC purification was performed with instruments the MAPS-100 system (Bio-Rad). All buffer

were prepared with sterile and free-pyrogen distilled water. All purified samples were extensively dialyzed against PBS, pH 7.4, and after appropriate dilution were sterilized by filtration on a 0.22 μm membrane (Millipore). Purified samples were aliquoted under sterile conditions and stored at -70°C.

Ammonium Sulfate Precipitation

The ascitic fluid was centrifuged at 100,000×g for 20 min at 4°C, and precipitated with a 33% saturated ammonium sulfate solution adjusted to pH 7.0. After 24 h, the sample was centrifuged at 10,000×g for 20 min at 4°C, and the pellet was resuspended in a small volume of water. The solution was then dialyzed against running water for 3 h to remove excess ammonium sulfate and finally dialyzed against PBS, pH 7.4.

Protein A High Performance Liquid Chromatography

The purification of McAb 3H11 from ascitic fluid was performed on a 100×25 mm affinity protein A column (Bio-Rad). The solution obtained after ammonium sulfate precipitation was diluted 1:1 with Affi-prep MAPS II binding buffer and loaded on to the column at a 5ml/min flow rate. Bound IgG were eluted with Affi-prep MAPS II elution buffer. The eluted peak was neutralized with 1 M Tris, pH 8.5, and then extensively dialyzed against PBS.

HPHT High Performance Liquid Chromatography

The McAb 3H11 obtained after ammonium sulfate precipitation was dialyzed against 10 mM PB, pH 6.8 and applied to a 50×25 mm HPHT column (Bio-Rad). Column loading was carried out with 10mM PB, pH6.8. IgG elution was obtained by running a 90 min linear gradient from 10-300 mM PB at a flow rate of 4 ml/min.

Protein A+HPHT High Performance Liquid Chromatography

Protein A purified McAb 3H11 was loaded on to HPHT column, column was operated the same conditions as over HPHT chromatography.

Protein Assay

The protein content in crude ascites fluid, purified ascites fluid or column fractions was determined using the microassay (Bio-Rad) with bovine plasma IgG as a standard.

SDS-PAGE

SDS-PAGE was performed according to Laemmli, using reducing conditions to separate light and heavy IgG chains. Stacking gels were prepared with 3% acrylamide, running gels with 12.5% acrylamide. Electrophoresis was carried out at a field strength of 5V/cm, gel were stained in Coomassie brilliant blue and destained in 10% acetic acid and 35% methanol in water.

Isoelectric Focusing (IEF)

IEF analysis was performed on a Mini IEF Cell (Bio-Rad) with containing a pH gradient from 3 to 10. Electrophoresis staining was carried out according to the instructions of the manufacture.

Immunoreactivity

Immunoreactivity was evaluated using ELISA. Target cell were immobilized on 96 well plates, after washing three times, purified McAb was added and incubated for 1 h at room temperature, after washing thoroughly, HRP-Goat anti-mouse IgG (1:2000) and a substrate solution containing OPD and H_2O_2 were added sequentially with 30 min incubation and washing between two addition. The reaction was terminated by the addition of 12.5% H_2SO_4 and the color intensity was measure at 492nM with on Elisa Reader (Bio-Rad 450). Normal mouse serum dilution to 1:1000 was run in parallel a negative control.

RESULTS

Pattern of Assay

Protein A HPLC obtained with mouse ascites containing McAb 3H11 are shown in Figure 1. Albumin and other impurities do not bound to protein A and elute with the binding buffer wash (Peak1), Protein A-bound IgG (Peak2) was eluted with elution buffer. HPHT HPLC was shown the presence of four peaks in Figure 2. Each one of these peaks was tested by ELISA. It was found that peak 1 was inactive, peak2 and peak3 was mildly active and peak4 was fully active. Protein A+HPHT chromatography pattern was shown in Figure 3. Notice that only single peak was clearly visible.

Purification Yields

Purified McAb 3H11 was checked by ELISA for IgG content. A value of 5mg/ml was obtained. Percent yields reported in Table 1 were calculated considering the actual amount of IgG recovered at the end of the entire purification process, including precipitation, centrifugation, dialysis and other steps. To achieve a higher degree of purify, only sharp peaks were collected.

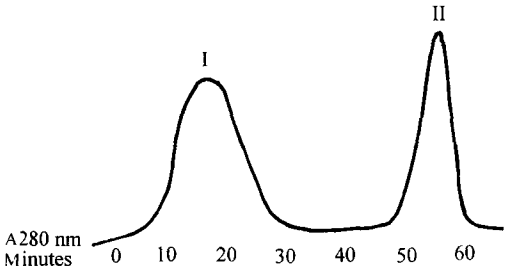

Fig. 1. The pattern of ascites containing McAb 3H11 with MAPS-100 system (100×25 mm protein A column)

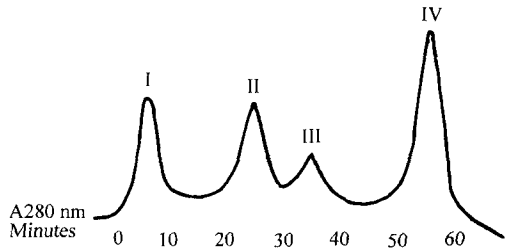

Fig. 2. The pattern of ascites containing McAb 3H11 with MAPS-100 system (30×4.6 mm HPHT column)

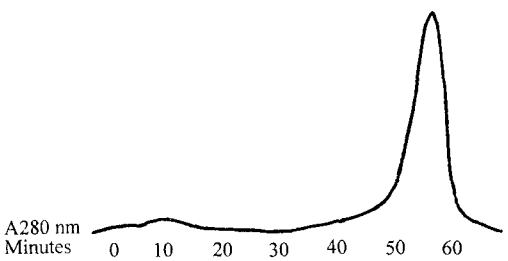

Fig. 3. The pattern of ascites containing McAb 3H11 with MAPS-100 system (protein A+ HPHT column)

Table 1. Purification yields

Purification protocol	Yield (%)
Protein A	48
HPHT	52
Protein A+HPHT	41

Purity

Purified McAb 3H11 was first checked for purity by SDS-PAGE (Figure 4) samples were performed under reducing and non-reducing condition. Under reducing condition, three methods purified products heavy and light can be discerned, HPHT purified product had trace amounts high molecular weight contaminants. However, under non-reducing condition protein A+HPHT purified sample had only one band. IEF result showed (Figure 5) that McAb 3H11 display three closed bounds, evidently different with purified polyclonar mouse IgG.

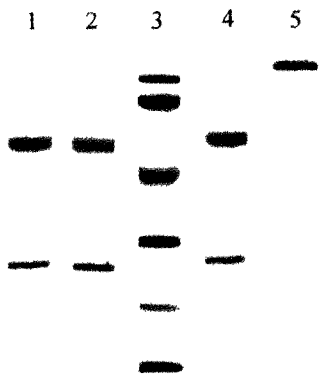

Fig. 4. SDS-PAGE of peaks collected after different methods purification of McAb 3H11
Lanes: 1, 2 and 4 Protein A, HPHT and Protein A+HPHT purified McAb 3H11 (Reducing condition)
Lanes: 3 Protein marks (14 KD-94KD)
Lanes: 5 Protein A+HPHT purified McAb 3H11 (Non-reducing condition)

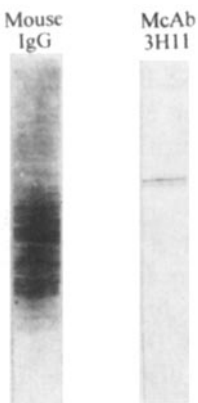

Fig. 5. Agarose isoelectric focusing electrophoresis, lower pH 3.0 and upper pH 10

Immunoreactivity

An ELISA was used to test binding activity of McAb 3H11 active peaks for three purification methods as shown in Figure 6. This result indicates that the McAb 3H11 isolated by protein A+HPHT two step purification is highly activity.

DISCUSSION

Staphylococcal protein A is well know for its property of binding the Fc part of IgG and is therefore widely used for isolating and fractionating IgG from mammalian sera and also for McAb purification. One of the great advantages of protein A affinity chroma-

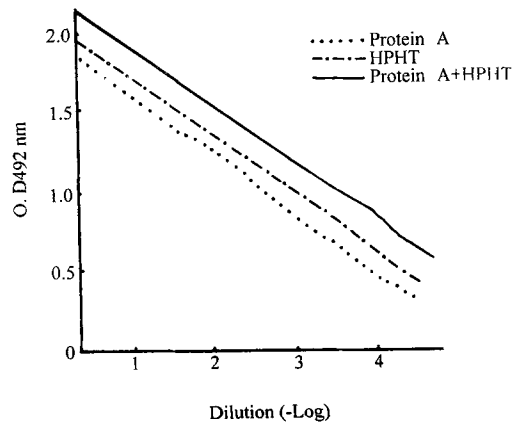

Fig. 6. The binding activity of McAb 3H11 peaks for three purified methods as indicated Figure 1 (II), Figure 2 (I) and Figure 3.

tography in McAb purification is the general applicability and the high selectivity for the IgG class, through which nearly 100% pure IgG can be obtained in one single step. However if McAb are purified from ascites, host IgG from the mouse will be co-purified and bovine IgG from in vitro culture supernatants may also bind to protein A. In addition, protein A has been claimed to be unsuitable for biopharmaceutical purification, since some leakage of the column can occur. Accordingly, a second purification step is necessary to separate McAbs. HPHT allow separation of a McAb from the host non-specific IgG contained in an ascites fluid, it was possible to achieve a high enrichment in specific IgG with the HPHT step. If minimal protein A leakage occurs, HPHT may discriminate IgG from a protein A-IgG complex.[5,6]

An analysis of data reported in table 1 showed the double step protein A+HPHT method to be the best purification procedure. This protocol satisfies every purity requirement and provides an IgG sample equivalent to affinity purified IgGs.

The protein A +HPHT methodology for the purification of McAb intended for in vivo use presents many advantages over the other procedures. It provides IgG with a degree of purity and is equally suitable for different IgG classes and subclasses. It is not time consuming, since the use of HPLC reduces run time to just a few hours.

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