PREPARATION OF CHLORAMPHENICOL ARTIFICIAL IMMUNOGEN AND MONOCLONAL ANTIBODIES

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ABSTRACT
Chloramphenicol (CAP) was coupled with bovine serum albumin (BSA) to prepare CAP artificial immunogen by mixed anhydride (MA) reaction, diazotization reaction and carbodiimide reaction whose reaction conditions were then further optimized. The successful linkage of the artificial immunogen was identified by UV Spectrophotometry (UV) and SDS Polyacrylamide gel electrophoresis (SDS-PAGE). Mice were immunized with different CAP artificial immunogen to obtain serum. Results determined by enzyme-linked immunosorbent assay (ELISA) showed that the CAP artificial immunogen synthesized by diazotization had the highest serum titer. Hybridomas were obtained by fusing mouse myeloma cells SP2/0 with splenocytes from the immunized mice. Hybridomas 2D1 secreting antibodies against CAP were obtained and subcloned. Ascites of monoclonal antibodies (McAb) were prepared by intraperitoneal injection of $1 \times 10^6$ cells of hybridomas 2D1 into mice abdomen. McAb obtained from hybridomas 2D1 were highly specific because there was no cross-reactivity with the drugs which were similar to CAP in their molecular structure.

Materials and Methods
Reagents and apparatus
BSA and OVA were obtained from Sigma (St. Louis, MO, USA). CAP was obtained from Aladdin (Shanghai, China). BALB/c mice were obtained from Zhejiang animal experiment center (Hangzhou, China). SP2/0 myeloma cell lines were obtained from Nanking KeyGen BioTech. Co., Ltd (Nanjing, China). Succinic Anhydride and N,N-dimethylformamide (DMF)
were obtained from Shanghai chemical reagent (Shanghai, China). Isobutyl Chloroformate, Dicyclohexylcarbodiimide (DCC), 1-hydroxy-1H-pyridine-2, 5-dione and Thiamphenicol were all obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Peroxidase-labelled goat anti-mouse IgG (HRP-IgG) was purchased from Boster Biotech Co., Ltd. (Wuhan, China).

UV-vis spectrophotometer (UV-1800) was purchased from SHIMADZU (Japan). Mini-PROTEAN Tetra Electrophoresis System 165-8001 was purchased from BIO-RAD (USA). Rotavapor R-210 was purchased from BUCHI (Switzerland). SpectraMax M2 was purchased from Molecular Devices (USA). AKTA purifier was purchased from GE Healthcare (USA). Cell culture plates (24 and 96 wells) and culture flasks were purchased from Costar Inc. (Cambridge, USA).

Buffer solutions used in the study were: coating buffer-20 mmol/L phosphate buffer (pH 7.4); washing buffer-10 mmol/L Tris-HCl (pH 7.4) containing 0.05 % Tween-20 and 0.8 % NaCl; blocking buffer-100 mmol/L PBS-T (washing buffer) containing 1% BSA; Substrate solution-50 mmol/L citric acid 14.58 ml, 0.2 mmol/L NaHPO4 7.71 ml, dH2O 7.71 ml, 30% H2O2 45 μl and O-phenylenediamine 12 mg.

**Preparation and detection of chloramphenicol immunogen**

1. **Mixed Anhydride (MA)**

Chloramphenicol hemisuccinate (CAP-HS) was synthesized by the method described by Schnappinger P. et al. (22, 28): 3 g of CAP and 500 mg of Succinic Anhydride were dissolved in anhydrous pyridine (reflux pipe connected CaCl2, drying tube and maintained the reaction of anhydrous and anaerobic) and stirred at 70°C for 6 h. After the reaction, the pyridine was removed by rotary evaporator and red sample was received. The reaction conditions were optimized. The samples were dissolved in 50 ml of ethyl acetate and 10 ml 0.1 mol/L hydrochloric acid then oscillated fully and the water phase was removed. Ten ml 10% NaHCO3 conversion solution was added, the pH value was adjusted to 3 with concentrated hydrochloric acid; the acid layer was discarded and washed with distilled water. The samples were dissolved in ethyl acetate and evaporated by rotary evaporator. CAP artificial immunogen (CAP-HS-BSA) was prepared as follows:

A solution-A total of 127 mg of CAP-HS was reconstituted with 10 ml N,N-dimethylformamide, stirred at 4°C for 10 min after mixing with 78.6 μl Tributylamine. The reaction mixture was added with 45 μl of isobutyl chloroformate, stirred at 20°C for 1 hour.

B solution-300 mg of BSA was reconstituted with 10 ml N,N-dimethylformamide, adjusted to pH 8.5 at 4°C. Concurrently, the B solution was added slowly to the A solution with stirring, the reaction mixture was allowed to incubate for 6 h at 4°C, while the pH of the solution was maintained at 8. The mixture was chromatographed on a Sephadex G-25 column, stored at -20°C after freeze-drying (Fig. 1).

2. **Diazotization**

500 mg of CAP were dissolved in 15 ml absolute alcohol in a stoppered round-bottom flask. The pH value of the solution was adjusted to 1 with hydrochloric acid, and 400 mg of zinc powder was added to it. The reaction mixture was stirred at 4°C with pH 1-2. Concurrently, 1 mol/L NaNO2 solution was dropped into the reaction mixture until the potassium iodide-starch test paper turned into blue (6). 500 mg BSA was dissolved in 10 ml PBS (20 mmol/L, pH 8) to form a BSA solution. The CAP solution with pH value of 8.0 (If Brown precipitate appears, the supernatants must be collected by centrifugation) was added to the BSA solution slowly, the reaction mixture was then stirred at 4°C for 6 h, and maintained at pH 8. Purification was achieved by gel filtration using a Sephadex G-25 column with 10 mmol/L PBS solution, then stored at -20°C after freeze-drying (Fig. 2).

3. **Carbodiimide**

Preparation of CAP-HS was performed after the same method as described above for Mixed Anhydride.

CAP-HS-BSA was synthesized by the method described by Arnold et al. (1) as follows: 136 mg of CAP-HS was dissolved in 50 ml of DMF, followed by the addition of 66 mg of DCC and 35 mg of N-hydroxy succinimide. The reaction mixture was incubated with stirring for 6 h at room temperature. The reaction mixture was centrifuged for 10 min at 4000 rpm, and the supernatant was collected. 240 mg of BSA was dissolved in 12 ml of PBS (10 mmol/L, pH 7.4), and 3 ml of the CAP-HS solution prepared above was added. The resulting mixture was reacted for 4 h with stirring at 4°C. Purification was achieved by gel filtration using a Sephadex G-25 column with 10 mmol/L PBS solution, then stored at -20°C after freeze-drying (Fig. 3). All of the reaction conditions were optimized.
4. Detection of CAP immunogen by UV
Solutions of the CAP immunogen (CAP-BSA, CAP-HS-BSA), standard BSA, and standard CAP were prepared in PBS buffer (10 mmol/L, pH 7.4), and scanned from 200 nm to 400 nm by ultraviolet photometric detector. The migration of maximum UV absorption peak could demonstrate that the coupling is successful, and then the coupling rate of artificial immunogen was calculated (2, 16).

5. Detection of CAP immunogen by SDS-PAGE
The CAP immunogen and standards were dissolved in PBS, and ultrafiltrated to remove the precipitation (4). The concentration of separation gel and stacking gel was 10% (m/v) and 4% (m/v), respectively. The sample concentration was 5 mg/ml, and the injection volume was 10 μl.

Preparation and Assessment of antibodies

1. Immunization
Each of six 8-week-old female BALB/c mice, was immunized with 100 μg of different CAP immunogen emulsified in Freund’s complete adjuvant. The immune response was strengthened three times at an interval of every two weeks, as mice were injected with the same antigen emulsified in incomplete Freund’s adjuvant. Blood was collected one week after the final immunization. The blood was allowed to clot at 37°C for 30 min (21, 27). The serum was collected by centrifugation and stored at -20°C.

2. Assessment of serum by indirect ELISA
The coating antigen synthesized by diazotization, CAP-OVA (100 ng/ml, 100 μl/well) was added to wells of enzyme label plate and incubated at 4°C overnight. Negative serum was added into last column wells as the control group while to the other wells different dilution of samples were added and incubated for 1 h. Further the second antibody was added to each well and incubated at 4°C for 1 h (9, 23). In order to determine the ELISA serum titer, the absorbance of each well was detected at 490 nm.

3. Preparation of McAb
The mouse with the highest serum titer was choosen, splenocytes were isolated from the spleen and hybridized with SP2/0 myeloma cells at a ratio of 5:1 by 50% polyethylene glycol. Fused cells were suspended in RPMI 1640 Medium that contained 20% FCS and HAT, added to the 96-well tissue culture plates, and cultured in 5% CO₂ incubator. One week after incubation, the hybridomas were screened by indirect ELISA and the positive hybridomas were re-screened by indirect competitive ELISA with CAP as the competitor. The hybridomas that excreted specific antibodies to CAP were subcloned by the limiting dilution method. The hybridomas producing McAb against CAP were transferred from the culture in the 96-well plate to a 24-well plate. After hybridomas became dense in the 24-well plate, they were transferred to cell culture flasks. Hybridoma cells were collected, centrifuged, propagated and stored in liquid nitrogen.

McAb against CAP was prepared in large scale by preparation of ascites in vivo. One week after intraperitoneal injections with 0.5 ml liquid paraffin, 8-week-old mice were planted with 1×10⁶ hybridoma cells by intraperitoneal injection. One week later, the ascites was collected as many times as possible.

4. Assessment of ascites
Ascites ELISA titers were obtained according to the indirect ELISA method as described above.

The cross-reactivity of ascites McAb and similar drugs (thiampenicol, florfenicol, penicillin, streptomycin, etc.) was tested by indirect competitive ELISA (ciELISA), which was determined by measuring their IC₅₀ values in the ciELISA using the midpoint of the CAP standard curve in all cases (3, 5, 8). The IC₅₀ values (50% inhibition levels) were used for calculation of cross-reactivity (CR₅₀, %) as follows: CR₅₀%= (IC₅₀ value of CAP/IC₅₀ value of competitor) ×100%.

Results and Discussion

UV scanning results
As shown in Fig. 4, Fig. 5 and Fig. 6, the λmax of BSA was 278 nm and λmax of CAP and CAP-HS were 277 nm. However, λmax of CAP-BSA (synthesized by diazotization) and CAP-HS-BSA (synthesized by MA and diazotization) were in the range of 274 nm-275 nm, and there is a hypsochromicly shift. The results indicated that the coupling of CAP artificial immunogen was successful. Besides, as shown in the figures, the maximum UV absorption peak of CAP-BSA and CAP-HS-BSA were significantly higher than the BSA at the approximate protein concentration, which also proved that the coupling was achieved.

SDS-PAGE results
As shown in Fig. 7, compared to the bands of BSA standard in lane 1, the bands of 6 antigens in lanes 2-7 obviously lagged behind, especially the antigens synthesized by diazotization in lanes 2 and 3. When the band lagged behind more obviously, this indicated that the combined ratio of CAP immunogen was greater, and it was consistent with the result from UV scans. Besides, there was a clear single band in every lane. This
showed that the CAP immunogen has reached electrophoretic purity and it is ready for preparation of antibodies.

**Fig. 4.** UV scanning of CAP immunogen (MA)

**Fig. 5.** UV scanning of CAP immunogen (Diazotization)

**Fig. 6.** UV scanning of CAP immunogen (Carbodiimide)

**Fig. 7.** SDS-PAGE of different CAP artificial antigen

Lane 1: BSA; lanes 2 and 3: diazotization reaction, coupling rate: 51, 39; lanes 4 and 5: mixed anhydride reaction, coupling rate: 9, 7; lanes 6 and 7: carbodiimide reaction, coupling rate: 18, 15; lane 8: marker

**Sensitivity of serum**

As shown in Table 2, CAP artificial immunogen synthesized by three methods all had good immunogenicity, especially the immunogen synthesized by diazotization reaction. The immunogen synthesized by MA was with the weakest immunogenicity.

**Table 1**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Coupling Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAP-BSA 1</td>
<td>51</td>
</tr>
<tr>
<td>CAP-BSA 2</td>
<td>39</td>
</tr>
<tr>
<td>CAP-HS-BSA 1</td>
<td>9</td>
</tr>
<tr>
<td>CAP-HS-BSA 2</td>
<td>7</td>
</tr>
<tr>
<td>CAP-HS-BSA(EDC) 1</td>
<td>18</td>
</tr>
<tr>
<td>CAP-HS-BSA(EDC) 2</td>
<td>15</td>
</tr>
</tbody>
</table>

**Table 2**

The highest dilution of different serum

<table>
<thead>
<tr>
<th>Methods, Coupling rate</th>
<th>The highest dilution of Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazotization, 51</td>
<td>1,562,500</td>
</tr>
<tr>
<td>Diazotization, 39</td>
<td>1,562,500</td>
</tr>
<tr>
<td>Mixed anhydride, 9</td>
<td>62,500</td>
</tr>
<tr>
<td>Mixed anhydride, 7</td>
<td>62,500</td>
</tr>
<tr>
<td>Carbodiimide, 18</td>
<td>1,562,500</td>
</tr>
<tr>
<td>Carbodiimide, 15</td>
<td>312,500</td>
</tr>
</tbody>
</table>

**Detection of hybridoma cell line**

After re-screened and subcloned four times by the limiting dilution method, the ratio of positive well was determined by ELISA (Table 3). As shown in the Table 4, Hybridoma cells of 2D1 can still stably secrete specific McAb against CAP.
TABLE 3

Result of Hybridoma Cell Cloning of Hybridoma Cell

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Times of clone</th>
<th>Growth well</th>
<th>Positive well</th>
<th>Percent of Positive well (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D1</td>
<td>1</td>
<td>53</td>
<td>45</td>
<td>84.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>71</td>
<td>71</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>63</td>
<td>63</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>68</td>
<td>68</td>
<td>100.0</td>
</tr>
</tbody>
</table>

TABLE 4

Specificity of McAb to CAP and its analogs

<table>
<thead>
<tr>
<th>Concentration of protein (mg/mL)</th>
<th>titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D1 cell supernatant</td>
<td>5.7</td>
</tr>
<tr>
<td>2D1 ascites</td>
<td>40.2</td>
</tr>
</tbody>
</table>

Monoclonal antibody sensitivity and specificity

Results presented in Table 4 showed the McAb was sensitive for CAP and Table 5 showed that the antiserum was specific for CAP. Although thiamphenicol and florfenicol have similar structure to CAP weak cross-reactivity was shown and there was no cross reaction between CAP antibody and other drugs.

TABLE 5

Specificity of McAb to CAP and its analogs

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cross-reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>100</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>0.07</td>
</tr>
<tr>
<td>Thiamphenicol</td>
<td>0.15</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Sulfamethazine</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Penicillin</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Neither carboxyl nor amino were found in CAP, but a contraposition aromatic nitro and an alcoholic hydroxyl groups exist. Therefore, there were two methods of synthesis of CAP artificial immunogen. One was aromatic nitro reduction to aromatic amino and connection with CAP by diazotization reaction; the other was succinic anhydride combined with terminal alcoholic hydroxyl by the method of MA/carbodiimide. Diazotization was a method which was widely used in chemical synthesis, referred to the reaction of primary aromatic amines with nitrous acid and gave diazonium salt. The reaction required strict experimental conditions, such as dark place and low temperature. Although the reaction conditions of MA are not strict, the operation process was quite tedious, time-consuming, and required some toxic reagents such as isobutyl chloroformate. Carbodiimide method was less time-consuming, but it might lead to protein denaturation because the reaction system lacked water.

Conclusions

This research proved that CAP artificial immunogen was successfully synthesized by three methods, and could satisfy the basic requirements as an immunogen. The combined ratio of diazotization method was the highest, which could reach a theoretical maximum of 50, while the combined ratio of MA method and carbodiimide method could only achieve 10 or so. Moreover, the reaction results of the latter two methods were unstable. Interestingly, the ELISA test results indicated that antibody titers corresponded with antigen and were almost the same when prepared by carbodiimide or diazotization. The reason may be the consumption of nitro on benzene ring in diazotization method which is important to maintain the specificity of CAP immunogenicity. However, taking into account all experimental conditions and the stability of the results, diazotization method can still be considered as the best synthesis method of CAP artificial immunogen. Furthermore, an ideal hybridoma cell line was successfully found, which could steadily secrete high sensitive and specific McAb of CAP.

Acknowledgements

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REFERENCES