Development of a Monoclonal Antibody to Detect αs1-casein in the Milk of Healthy and Mastitis-Affected Goats

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Abstract
This study aimed to evaluate the expression level of caseins during mastitis of goats. Whole goat caseins were used as primary antigens for mouse immunization. A monoclonal antibody (mAb) named 5B with high specificity to goat αs1-casein was developed. Further results showed that mAb 5B can successfully be applied to western blot and ELISA. In addition, immunofluorescence analysis using this mAb showed increased milk αs1-casein level in mastitis-affected goats. In conclusion, this study has established an effective tool to evaluate the expression level of αs1-casein during mastitis development.

Keywords: αs1-casein, Monoclonal antibody, ELISA, Mastitis, Immunofluorescence, Goat

INTRODUCTION
Goat milk and its productions begin gaining attention because of their easy digestibility and lower allergenic properties compared to the cow milk (1). An important role for goat milk is to provide the necessary nutrients for infants affected by cow milk allergy (2). In addition, the nutritional value of goat milk has been widely accepted considering the total protein, fat, vitamin and mineral contents (3,4). Caseins in milk account for more than 80% of total proteins and consist of αs1-, αs2-, β-, and κ-caseins and αS1-casein is one of the most important highly-phosphorylated proteins among caseins (5). Goat milk contains less αs1-casein (5%-20%) compared to (30%-35%) cow milk, making goat milk more similar to human milk and less likely to cause allergy (6). In addition, αS1-casein influences the coagulation properties of milk (7). With the development of hybridoma technology, monoclonal antibodies (mAbs) against caseins were used to determine the content of β-casein in bovine milk (8). However, up to the present, mAbs against goat caseins have not yet been developed.

In this paper, we used purified goat caseins for mouse immunization. Two strains of hybridoma cells targeting goat caseins (named 5B and 7H) were screened by indirect
ELISA. To explore the effect of mastitis on αs1-casein, we analyzed the level of αs1-casein of goat milk samples collected from healthy goats, goats with sub-clinical mastitis and clinical mastitis by indirect competitive ELISA. Immunofluorescence of goat mammary epithelial cells was carried out to further examine αs1-casein expression profile in lipopolysaccharide (LPS)-induced mastitis in vitro.

MATERIAL and METHODS

Ethical Approval

Animal-related experiments in the present study were approved by the Research Ethics Committee of Northwest A&F University according to the guidelines of the Ministry of Health in China for the care and use of laboratory animals.

Antigen (Whole Goat Caseins) Preparation

Goat caseins were extracted using isoelectric precipitation. Briefly, 10 g of whole goat milk powder (purchased from Kabtrita, Holland) was dissolved in 10 mL of 2M Na2HPO4•12H2O buffer. The pH was adjusted to 4.2 using 1M CH3COOH solution (preheated to 40ºC). After complete precipitation at 37ºC, the solution was centrifuged at 4000g for 10 min, and the precipitate was washed with 20 mL of 95% (v/v) ethanol 3 times to remove lipid. Next, 1:1 (v/v) ethanol-ether mixture was added to wash the precipitate 3 times. Final precipitation was washed twice with ether and was placed in a fume hood until all ether volatilized. Casein powder was preserved at -80 ºC and its purity and molecular weight were analyzed by SDS-PAGE [6].

Mouse Immunization, Cell Fusion, Hybridoma Cell Screening and Ascites Preparation

Mouse immunization, cell fusion, hybridoma cell screening and ascites preparation were conducted as previously reported by our group [6].

Competitive Indirect ELISA

Competitive indirect ELISA was conducted as previously reported [6]. Specifically, the dilution of goat milk was 15 fold with PBST and the dilution of mAb 5B was 7 fold with PBST.

Milk Sample Collection and Processing

For clinical mastitis samples, milk with the detection of flakes and clots with gland swelling or systemic illness such as fever, depression, weakness and dehydration were selected. For sub-clinical mastitis diagnosis, the California Mastitis Test was used as previously reported [7]. All the milk was stored at 4ºC after collection and was used for further assays within 1 h.

Isolation and Culture of Goat Mammary Epithelial Cells (MECs)

Goat MEC isolation was performed as previously reported [6]. After isolation, the cells were cultured in DMEM/F12 medium (Thermo Fisher Scientific, New York, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), 1% GlutaMAX Supplement (Thermo Fisher Scientific) and 100 units mL-1 of penicillin-streptomycin (Sigma Aldrich, Missouri, USA). The culture medium was refreshed every 24 h. The cells were cultured at 37ºC in 5% CO2 and were passaged when they were 80% confluent.

LPS Stimulation and Immunofluorescence Staining

The in vitro mastitis model was established by adding LPS (Sigma Aldrich) to the culture medium at a final concentration of 10 μg mL-1. Meanwhile, the control group was treated with culture medium of the same volume. All cells were treated for 12 h before immunofluorescence analysis. Procedures of immunofluorescence were the same as previously reported [8].

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). Differences were assessed using t-tests or one-way ANOVA with Dunnett test. P<0.05 was considered significant.

RESULTS

Isoelectric point precipitation method was used for the purification of the goat casein and was identified by SDS-PAGE. After precipitation, caseins were purified and contained αs1-casein because lane 3 and lane 4 has an identical band at 23.9 kDa (Fig. 1). Consistent with a previous study, purified αs1-casein showed a higher molecular weight when analyzed by SDS-PAGE than expected [9].

The titers of sera collected from the immunized mice 7-10 days after the forth immunization were determined by indirect ELISA. All immunized mice produced anti-goat

![Fig 1. SDS-PAGE analysis of caseins. Lane 1, bovine milk powder; lane 2, goat milk powder; lane 3, total goat caseins; lane 4, goat αs1-casein; lane 5, bovine α-casein](image-url)
casein antibodies. Several mice were selected for further experiment and were injected with 100 μg of immunogen intravenously 3 days before cell fusion. Then, hybridoma secreting antibodies specific to goat caseins but unrecognizable to bovine milk powder were selected by indirect ELISA, and were subcloned by limiting dilution method. Two stable hybridoma cell lines were established and named as 5B and 7H. These two hybridoma cell lines showed strong proliferation ability and high specificity to goat caseins and was chosen for further studies.

Cross-reactivity of mAbs 5B and 7H was analyzed by indirect ELISA using goat casein, bovine milk, goat milk, goat milk powder and bovine milk powder as antigens. The results showed that these two mAbs both specifically recognized goat caseins and had no cross-reactivity with bovine caseins (Table 1), which was supported by western blot analysis using mAb 5B (Fig. 2A). In addition, the specificity of mAb 5B was further evaluated using different subtypes of goat caseins. The result showed that this mAb only reacted with goat αs1-casein, while no other reactions existed between mAb 5B and purified β- and κ-caseins (Fig. 2B).

In order to evaluate αs1-casein content in milk of goat with mastitis, OD450 values of different goat milk samples were compared by indirect competitive ELISA. As illustrated in Fig. 3A, milk from goats suffering from clinical mastitis had the lowest OD450 value, representing the highest levels of αs1-casein in these samples. In comparison, milk from goats with sub-clinical mastitis and healthy controls had relatively lower levels of αs1-casein compared to the goats with clinical mastitis (P<0.05). We further built an in vitro mastitis model by challenging goat MECs with LPS. Interestingly, LPS treatment resulted in significantly decreased cytoplasmic αs1-casein levels in MECs (Fig. 3B-C).

Table 1. Testing specificity of monoclonal antibodies 5b and 7b against goat casein by ELISA

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Goat Casein</th>
<th>Goat Milk</th>
<th>Goat Milk Powder</th>
<th>Bovine Casein</th>
<th>Bovine Milk</th>
<th>Bovine Milk Powder</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAb 5B</td>
<td>1.762</td>
<td>1.531</td>
<td>1.824</td>
<td>0.080</td>
<td>0.075</td>
<td>0.082</td>
</tr>
<tr>
<td>MAB 7H</td>
<td>1.634</td>
<td>1.520</td>
<td>1.627</td>
<td>0.092</td>
<td>0.065</td>
<td>0.087</td>
</tr>
</tbody>
</table>

Fig 2. mAb specificity tests by western blot analysis. (A) Goat casein was loaded as a positive control, the content of goat casein and bovine casein was 1.7 μg, milk powder was at the concentration of 0.1 g/mL, the milk was 20-fold diluted. Lane 1, marker; lane 2, bovine α-caseins; lane 3, cow milk; lane 4, cow milk powder; lane 5, total goat casein; lane 6, goat milk; lane 7, goat milk powder. (B) Isolation of goat caseins and recognition of 5B to αs1-casein as determined by western blot. Goat caseins are purified by cation-exchange chromatography. Each lane is loaded with the correspondingly labeled proteins.

Fig 3. Analysis of αs1-casein level in goat milk samples and goat MECs. (A) Indirect competitive ELISA analysis of αs1-casein content in milk samples from goats with clinical mastitis (n=10), sub-clinical mastitis (n=8) or healthy goats (n=6), (B) Immunofluorescence analysis of αs1-casein expression levels in goat MECs with (LPS, n=4) or without LPS (Control, n=4) treatment. (C) Integrated optical density analysis of the immunofluorescence results in (B).
DISCUSSION

Casein expression level is an important index when evaluating the lactation function of the mammary gland. While mAbs against β-casein [10] and κ-casein [11] have been developed, no studies have reported the successful development of mAbs against αs1-casein, the major part (38%) of total caseins in milk [12]. In addition, considering that αs1-casein is the most important protein causing milk allergy [13], mAbs with high specificity to goat αs1-casein are therefore of critical importance for people to avoid milk allergy.

Mammary epithelial cells constitute an important part of the mammary gland and are the structural basis for the lactation of the mammary gland. The secretion of caseins is affected by the condition of MECs. By using mAbs against αs1-casein, we can measure its expression level in MECs under different conditions. Intriguingly, our data suggest that the concentration of αs1-casein was significantly increased in milk samples of clinical mastitis-suffered goats, indicating that the milk of these goats may be problematic and chances of allergy are higher. However, we could not rule out the possibility that whether an enhanced αs1-casein secretion ability accounted for less αs1-caseins retention. Thus, future studies are needed to further explore the underlying mechanisms.

In conclusion, a mAb named 5B that specifically reacted with goat αs1-casein was developed in the present study. This mAb can successfully be applied to western blot, ELISA and immunofluorescence and served as a convenient tool to assess the dynamics of αs1-casein during mastitis development. More future studies investigating the biological function of αs1-casein and its relationship with certain diseases are encouraged. Specially, studies concerning the involvement of αs1-casein in milk allergy will lay important foundation for elucidating the underlying mechanisms and for effective therapy development.

CONFLICT OF INTEREST

The authors declare that no conflict of interest exists.

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