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Isolation and Preparation of Monoclonal Antibody to Ovine Immunoglobulin M

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Abstract: Ovine immunoglobulin M was isolated from sheep serum using gel filtration and affinity chromatography. Polyclonal and monoclonal antibodies against this isotype were prepared. The specificity of the monoclonal antibody was investigated using SDS-Page, immunoblotting, an enzyme-linked immunosorbent assay (ELISA) and affinity chromatography. On immunoblotting, monoclonal anti-IgM reacted with a 70 kD protein which corresponded to ovine IgM.

Key Words: IgM, Isolation, monoclonal, antibody.

Koyunlardan IgM izolasyonu ve monoklonal anti-IgM hazırlanması

Özet: IgM, jel filtrasyon ve afinite kromatografi yöntemleri ile koyun serumundan izole edildikten sonra poliklonal anti-IgM ve monoklonal anti-IgM hazırlandı. Monoklonal antikorların spesifitesi SDS-Page, immunoblotting, enzyme-linked immunosorbent assay (ELISA) ve afinite kromatografisi ile araştırıldı. Monoklonal anti-IgM, immunoblotting testinde 70kD ağırlığında bir protein ile reaksiyon vermiş ve bu proteinin IgM olduğu saptanmıştır.

Anahtar Sözcükler: IgM, izolasyon, monoklonal, antikor.

Introduction

Immunoglobulin M (IgM) is composed of five identical 180kD subunits with a molecular weight of 900kD, joined together by J chains (1, 2). It has been reported that the molecular weight of its heavy chain is 70-75kD (2). IgM in colostrum and milk is derived from the serum (1) and its serum concentration (1.51 mg/ml), is lower than that in the colostrum (7.1 mg/ml) (3, 4).

IgM is the major immunoglobulin produced in the primary immune response. The importance of the role of IgM in the protection of the mucosa in ruminants is less clear than it is important in monogastric species (4). However, bovine IgM can fix the complement by the classical pathway (5). Opsonization by IgM in newborn calves has been reported (6) and the bactericidal activity of serum and milk to mastitis pathogens often appears to be associated with IgM (7, 8).

Monoclonal antibodies have many advantages over polyclonal antisera in terms of specificity and affinity but polyclonal antisera are useful in techniques which involve precipitation reactions, such as immunodiffusion and immunoelectrophoresis. Polyclonal antisera need heavy absorption in order to render them specific and a very limited amount of antisera can be obtained. In contrast with polyclonal antibodies, specificity can be achieved through monoclonal technology and clones can be preserved for a long time. In addition, a high quantity of antisera can be collected from the clones.

A good, high-titered monoclonal anti-ovine IgM antibody must be used in experiments. The aim of this study was to isolate ovine IgM and prepare a monoclonal antibody against this isotype. Gel filtration and affinity chromatography with protamine sulphate were used in purification. The monoclonal antibody was produced using this preparation and the resulting hybrids were analysed for antibody production using an enzyme-linked immunosorbent assay.

In order to assess the specificity of the monoclonal antibodies, isotype specific affinity chromatography was carried out using the ovine monoclonal antiserum. The

bound proteins were eluted and analysed by immunoelectrophoresis and SDS-Page. The molecular weight of the epitopes recognized by this monoclonal antiserum was determined by immunoblotting.

Material and Method

Isolation of IgM

Two hundred ml of sheep serum was precipitated with 50% saturated ammonium sulphate solution. Serum samples were added to a sephadex G-200 column, as described by Yılmaz et al. (9). The first peak from the G-200 column was collected and added to a column containing protamine sulphate linked to Sepharose 4-B. The column was washed for 4-5 hours with 0.026M phosphate buffer containing 0.025M sodium chloride. Elution was performed with 0.08M phosphate buffer containing 1.1M sodium chloride (10). The proteins were eluted, concentrated, filtered and dialysed in PBS for 24 hours. Electrophoresis of the eluent against anti-ovine whole serum revealed 3 proteins two of which resembled IgM and α-2 macroglobulin, respectively, in their migration pattern. The preparation was then purified using affinity chromatography with sepharose bound anti-ovine α-2 macroglobulin. It was analysed by immunoelectrophoresis and SDS-Page. This preparation was used for immunization of mice to prepare the monoclonal antibody against this isotype. The same procedure was also used to prepare polyclonal antibody in rabbits, as described by Yılmaz et al. (9).

Protamine sulphate affinity chromatography

The immunoabsorbent was prepared according to the method described by Marc et al (11) and Yılmaz et al (9). The protamine sulphate was dissolved in PBS, pH 7.4, and linked to Sepharose 4B (Pharmacia, UK) 0.08M phosphate buffer. 1.1M sodium chloride and 0.02M phosphate buffer were added as the eluting and washing buffers respectively (10).

Determination of immunoglobulin concentration

The concentration of immunoglobulins was determined using spectrophotometry with an optical density of 280nm (Unican SP1800 Pye Cambridge). An extinction coefficient for 0.1% solution in a 1cm light path was taken as 1.4. This procedure was used to determine the concentration of IgM prior to the coating of ELISA plates and in preparing immunoglobulins for immunization and as standards in SDS-Page and immunoblotting.

Immunization of mice

The mice were injected intraperitoneally with 200-400 µl of Freund's complete adjuvant containing 50-80 µg protein. They were then boosted with the same amount of proteins in Freund's incomplete adjuvant, at 14 day intervals until a good titer was obtained. Blood samples were taken from the tail of the mice after 2-3 injections and antibody titers were analysed by ELISA. Mice exhibiting antibody titers of 1:1000 were boosted intraperitoneally four days before fusion with the same amount of antigen without adjuvant.

Preparation of monoclonal antibody

A modified version of the method of Galfare et al. (12) was used in this study (13). The immunized mice were outhanasied and $2.5x10^7$ myeloma cells (P3x63 AG 8.653 or P3 NS1) were fused with $1x10^8$ immunized spleen cells, after washing three times with serum-free RPMI-1640 (Flow Laboratories). The supernatant was carefully discarded and 0.5 ml of prewarmed polyethylene glycol 1500 (PEG 50%, w/v in 75m Hepes, Boehringer) solution was added drop by drop over 1.5 minutes with continuous shaking. 10 ml of prewarmed RPMI-1640 was then added gradually and completed in a 5-minute period. The mixture was centrifuged at 350 g for 5 minutes and the cells resuspended in 40 ml of complete medium and allowed to stand at 37°C for 10-20 minutes. After centrifugation, the cells were transferred to 96 well plates, in selective HAT (Flow Laboratories) RPMI-1640 medium, containing 15% fetal calf serum supplemented with 2mM glutamine, 1mM sodium pyruvate, 2.5 µg amphotericin B, 50 µg streptomycin and 50 IU penicillin. Growth-promoting factors, obtained from normal mouse spleen cells through incubation in 50 ml of this medium for 2-3 days, were also added. The spleen cell supernatant was added to fresh medium in a ratio of 1:3. The cells were fed with HAT RPMI-1640 medium containing growth factors by removing half the volume of the medium every 2-3 days. After 10 days, the medium was replaced by hypoxanthine thymidine (HT) medium.

The hybridoma cell culture supernatants were analysed by ELISA 12-15 days after fusion. Cells from the fusion, secreting specific antibody against ovine IgM, were cloned by limiting dilution, at least twice or until monospecificity was achieved. This was evaluated by the occurrence of specific antibody secreting clones in all the wells containing hybrid cells.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA plate (Greiner) was coated with 100 µl of 5-640 ng/ml of purified ovine IgM, in coating buffer (pH 9.6) containing 1.59 g sodium carbonate anhydrous and 2.93 g sodium hydrogen carbonate in 1000 ml distilled water and incubated at 4°C overningt or for 2 hours at 37°C. The plates were then washed with PBS containing 0.05% Tween-20 and incubated with culture supernatants from the fusion (monoclonal anti-ovine IgM) for 2 hours at room temperature. The plates were incubated with 100 µl of an alkaline phosphatase conjugated sheep anti-mouse IgG antibody (Sigma) for 1 hour after washing with PBS Tween-20. It was washed again with PBS Tween-20 and the color was developed with 100 µl of 1mg/ml phosphatase substrate (P-Nitrophenyl phosphate, disodium, hexahydrate, Sigma Ltd.) in coating buffer.

In titrating out the monoclonal antibodies, the plates were coated with doubling dilutions of purified immunoglobulin running in a horizontal direction. Monoclonal antibody, starting at a dilution of 1:50, was then added in doubling dilutions running in a vertical direction.

SDS-Page

This was carried out as described by Laemli (14). The test preparations were mixed with sample buffer containing 0.5 M, 25% (v/v) Tris pH 6.8, 1% (w/v) sodium dodecyl sulphate and 2.5% (v/v) β_2-mercaptoethanol. These were heated at 95-100°C for 5 minutes. The samples and SDS-6H molecular weight markers (Sigma) were run on one half of the gel and the sequence repeated on the other half. Electrophoresis was performed with running buffer containing 9 g Tris, 37.5 g glycine and 3 g sodium dodecyle sulphate in 3 litres of distilled water. The proteins in the gel were transferred to a nitrocellulose paper, 30 V, overnight in blotting buffer containing 9 g Tris, 45 g glycine and 600 ml methanol in 3 litres of distilled water. The first half of the membrane was stained with 1% (v/v) amido black staining, and the second half was used for immunoblotting.

Specificity of the monoclonal

a) Immunoblotting

Immunoblotting was performed as described by Towbin et al. (15). The nitrocellulose membrane was incubated with culture supernatant obtained from mouse anti-ovine IgM fusion at a dilution of 1:100 for 2 hours at room temperature, after blocking of the remaining binding sites with 5% (w/v) dried fat-free milk (Marvel) in PBS. After washing with PBS containing 0.05% Tween-20 (v/v), the membrane was incubated with peroxidase conjugated sheep anti-mouse immunoglobulins (Sigma) at a dilution of 1:1000, for 2 hours at room temperature. Washing was repeated in PBS Tween-20. Finally, the membrane was developed with 1 mg/ml 3,3-diaminobenzidine (Sigma) in PBS containing 0.01% hydrogen peroxide.

b) Affinity chromatography

The culture supernatant was bound to Sepharose-4B for affinity isolation (after the results were obtained from the immunoblotting as it gave one band on the IgM region) to prove that the immunoglobulin in the supernatant had reacted with the IgM. The product obtained from the affinity isolation was used in immunoelectrophoresis.

Freezing of hybridoma cells

$2x10^6$ cells producing monoclonal antibody against ovine IgM were frozen in 0.5 ml of cryopreservant (10% dimethyl sulfoxide, 90% fetal calf serum). The cells were centrifuged at 350g for 5 minutes and the cell pellet was resuspended in 1 ml cryopreservant. These cells were kept at -70°C for 20-24 h and then transferred to liquid nitrogen for storage until required.

Results and Discussion

In the present study, ovine IgM was prepared from serum rather than colostrum to avoid the problem of contamination with IgA, although the concentration of IgM in serum is lower than in colostrum (4). IgM was prepared by gel filtration and most of the contaminating α-2 macroglobulins were removed using affinity chromatography, resulting in a pure preparation of Igm.

The polyclonal anti-ovine IgM was prepared in rabbit using this preparation and the antiserum absorbed with an IgG fraction of G-200 column chromatography in order to remove anti-light chain activity. This antiserum, after absorption, produced one electrophoretic arc in the region characteristic for IgM in the preparation of affinity isolated proteins from α-2 macroglobulin and the monoclonal anti-ovine IgM affinity column (Figure-1). SDS-Page analysis of this preparation revealed proteins in the regions of 22 and 70kD, which are similar to the light chain and heavy chain of bovine IgM (2).

The monoclonal antibody to ovine IgM was produced after 4 fusions. Supernatants from 2 hybrid cell clones were positive for anti-ovine antibody. One of these clones was positive for anti-ovine IgM. It was cloned and recloned to achieve monospecificity and expanded to a

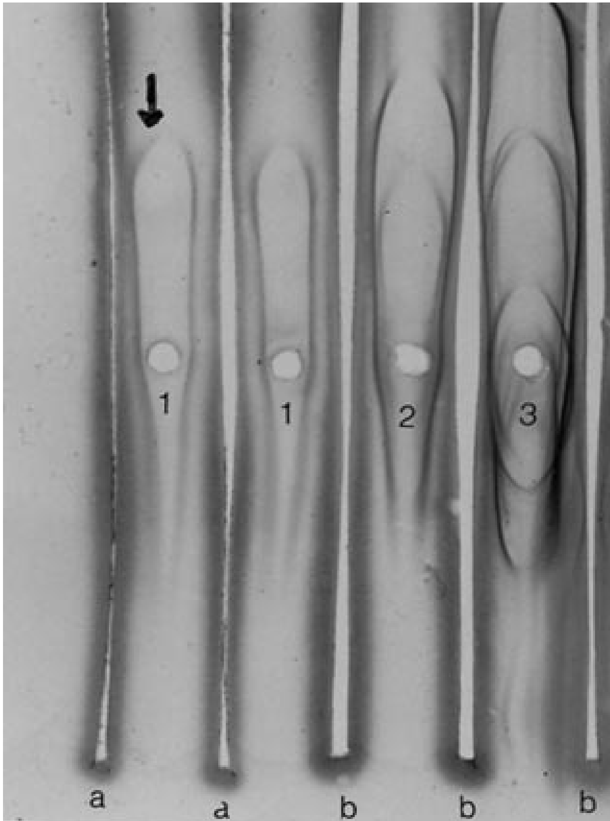

Figure 1. Immunoelectrophoresis of ovine IgM, G-200 fraction of sheep serum and sheep serum using polyclonal anti-ovine IgM and anti-ovine whole serum. Arrow indicates the precipitin line of IgM.
1. Affinity-isolated IgM from monoclonal anti-ovine IgM immunoabsorbance.
2. IgM preparation after absorption with anti-ovine alpha-2 macroglobulin immunoabsorbance.
3. First peak from G-200 chromatography of sheep serum.
a. Rabbit anti-ovine IgM serum.
b. Rabbit anti-ovine whole serum.

100 ml culture. The supernatant from the expanded hybridoma cells showed low optical density with ELISA.

The second and third attempts to prepare monoclonal antibody to ovine IgM also resulted in a low-titered monoclonal antibody and the hybrids ceased producing anti-ovine IgM antibody. There may be a number of reasons for these 3 unsuccessful fusions.

The aim of the immunization of the mice was to produce the maximum number of clones secreting specific antibody. The more numerous the anti-IgM producing B cells are, the higher the chance of fusing these cells with myeloma cells (16). In the present study, the immunization was similar to that described by Campbell (17). It produced an antibody titer of 1:1000 and each fusion of 10^7 lymphocytes resulted in the production of 10-70 antibody secreting hybridoma cells. This is similar to 1-100 hybrids for 10^7 lymphocytes, reported by Campbell, (17) and Pervez, (18). However, the ratio of myeloma to immunized spleen cells is also important in the fusion. In the present study, the ratio was 1:4 and 1:5. This is in agreement with that of Campbell (17) but is greater than the 1:1 ratio used by Beh (19) to produce anti-sheep IgG1. Results from 3 fusion, showed that there were mouse B cells secreting anti-ovine IgM but the success of the fusion is entirely dependent on the chance of fusing high antibody producing cells with myeloma cells. In these three fusions low antibody producing or negative cells may have fused with myeloma cells. It is less likely that it was the result of technical errors. Positive cells resulting from the fusion also stopped producing antibodies. This might be because of chromosomal changes during culture (16).

The fourth fusion to prepare monoclonal anti-ovine IgM resulted in 6 positive hybridoma cell clones for anti-ovine antibody. Two of these hybridoma cells were positive for anti-ovine IgM antibody. Hybridoma cells were cloned 4 times to achieve monospecificity and the supernatants were screened by ELISA 10 days after every cloning. It was then expanded to a 50-100 ml media and the supernatant was collected. The supernatant was titrated by ELISA for antibody titer and species specificity. The monoclonal antibody gave maximum optical density at a dilution of 1:800 with 500 ng/ml of antigen coated on the ELISA plate. The end point titer was 1:1600 with an antigen concentration of 64 ng/ml. Monoclonal antibody also showed cross-reactivity with bovine (kindly provided by D. Patel, Langford, Vet. School U.K.) and buffalo IgM (kindly porvided by Dr. K. Pervez, Langford, Vet. School U.K). The control, wells antigen-coated and uncoated, were negative. The hybridoma cells producing anti-ovine IgM were harvested and frozen till required.

The monoclonal antibody was bound to sepharose 4-B in preparation for affinity chromatography. Amido black staining of the IgM prepared using affinity chromatography of monoclonal anti-ovine IgM revealed 2 bands in the region of 22 and 69-70 kD (Figure-2), which are identical to the human light and heavy chain IgM respectively. However, the molecular weight of the heavy chain for bovine IgM, 75-78 kD (2, 20), and buffalo IgM heavy chain, 82 kD (18), are slightly larger than the ovine IgM heavy chain reported in this study.

The monoclonal antibody reacted with a single protein band in the region of 70 kD when used in immunoblotting (Figure-2). This corresponds to the

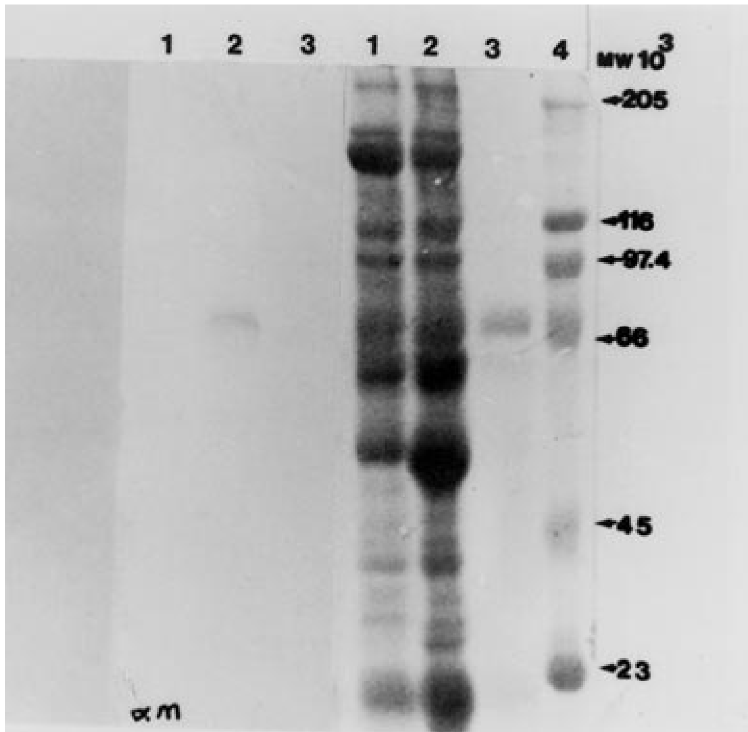

Figure 2. Immunoblotting with monoclonal anti-ovine IgM.
The right-hand side shows amido black staining of proteins transferred to nitrocellulose paper:
1. G-200 fractions of specific pathogen-free sheep serum.
2. G-200 fractions of normal sheep serum.
3. Affinity-isolated IgM from monoclonal anti-ovine IgM immunoabsorbance.
4. SDS-6H molecular weight markers.
The left-hand side shows immunoblotting with monoclonal anti-ovine IgM:
1. Semipurified ovine IgA
2. G-200 fractions of normal sheep serum
3. Purified ovine IgG

protein band stained with amido black staining in the region of 69-70 kD suggesting that it was ovine IgM heavy chain. There was no reaction against ovine IgG, IgA and serum proteins in sheep serum.

These findings suggest that the immunoglobulin isolated from ovine serum using gel filtration and affinity chromatography is ovine IgM. Immunoblotting with monoclonal anti-ovine IgM showed that the molecular weight of the heavy chain of this isotype appears to be 69-70 kD. Further investigations are necessary to understand the function of this isotype in the immune system of sheep.

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