Monoclonal antibodies against bovine growth hormone

Pradeep Kumar & Yudhisthir S. Rajput*

Animal Biochemistry Division, National Dairy Research Institute, Karnal 132001, India

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A number of mouse x mouse hybridomas producing monoclonal antibodies (MAbs) against bovine growth hormone (bGH) were prepared by fusion of spleen cells from bGH-primed mice (Balb/c) with non-secretory mouse myeloma cells (PAIOP3) and characterized. MAbs obtained from three fusion experiments belonged to IgM, IgG1 and IgG2b class/subclass of antibodies. Cross-reaction studies indicated that generated antibodies were against three different epitopes of bGH. VIIB6E8 (IgG1) and VIIB2E1C9 (IgM) did not cross-react with ovine prolactin (oPRL), ovine luteinizing hormone (oLH) and porcine follicle stimulating hormone. Antibody VIIB3C9E8 (IgM) exhibited cross-reaction with oPRL and oLH. Antibody VIIC1F8 (IgG2b) cross-reacted with oPRL. All MAbs were against conformational epitopes of bGH.

Bovine growth hormone (bGH) is a single chain polypeptide of 191 amino acids produced by anterior pituitary gland1. It is a single domain molecule that folds into a four antiparallel α-helix bundle2. bGH has been completely sequenced3 and it has two disulfide bridges, one connecting cystine residues at position 53 to 164 and other connecting cystine residues position 181 to 1894. These bridges configure the molecule into two protein loops. The major immunologically active zones are confined to 87-124 and 125-149 regions5. Somatogenic as well as lactogenic activities are present in 22 kD bGH. Although molecular events how bGH triggers these activities are not completely elucidated, but it has been observed that exogenously introduced bGH results in enhanced level of insulin like growth factors I (IGF-1)6, a molecule central to somatogenic as well as lactogenic response. These activities are not located in one portion of molecule suggesting distant amino acid residues might be involved in creating a site through which bGH binds to its receptor. The involvement of distant amino acid residues in an epitope is also known and an epitopic site may or may not overlap the site which binds to receptor. The antibodies can modulate activities of bGH depending upon to which epitope these bind. With the advent of development of protocol for monoclonal antibodies (MAbs) production of predefined specificity in 1975 by Kohler and Milstein6, MAbs against different epitopes of bGH can be generated. In the present investigation, it has been shown with the help of generated MAbs against bGH that there are at least three epitopes in bGH and all of them being conformational epitopes.

Materials and Methods

Balb/c mice and mouse myeloma cell line PAIOP3 (non-secretory) were obtained from Institute of Microbial Technology, Chandigarh. bGH was kindly provided by USDA-Animal Hormone Programme, USA.

Immunisation of mice—Balb/c mice (2 months old) were immunised intraperitoneally. Equal volumes of bGH (100 μg/100 μl PBS) and Freund's adjuvant were mixed. In each immunisation, 100 μg bGH was administered. The first injection was given in Freund's complete adjuvant (FCA) followed by two injection in Freund's incomplete adjuvant (FIA) at 15 days intervals. For booster injection, bGH (100 μg) dissolved in 200 μl PBS was given intraperitoneally for three consecutive days before removal of spleen for cell fusion.

Preparation of hybridoma—The cell fusions were performed according to standard method as described by Gallie and Milstein7. The sensitized mouse spleen cells were fused with PAIOP3 myeloma cells in 10:1 ratio using 50% polyethyleneglycol (Mw 4000 dalton). After fusion, the cells were suspended in a selective growth medium containing hypoxanthine, aminopterin and thymidine (HAT) and were cultured in 24 well plates at 37°C in humidified 5% CO2/95%.
The hybrids were regularly observed under inverted microscope and based on cell density, medium was replenished with HAT on around day 7. The cells were allowed to grow in HAT medium for atleast 10 days before these are shifted to HT medium. After 1 week of growth of cells in HT medium, cells were shifted to normal medium. At about half confluency, supernatants from growing hybridomas were screened for the presence of antibody using enzyme-linked-immunosorbent-assay (ELISA). The hybridomas producing anti-bGH antibody were cloned by limiting dilution method where peritoneal cells were used as feeder-layer. The clones were screened for anti-bGH antibodies in ELISA. Clones tested positive were selected and recloned if necessary. One such selected hybridoma was expanded in 25 cm² tissue culture flask and cells suspended in PBS were injected to pristane or FIA-primed Balb/c mouse for ascite production.

**ELISA**—ELISA was used for screening of hybridomas 96-wells flat bottomed polystyrene plates (Costar, USA) were used in ELISA and method described by Engvall and Perlman with some modifications was adopted. Briefly, multwell plates were coated with bGH (1μg/100 μl PBS pH 7.4/well) by overnight incubation at 4°C. All subsequent incubations were carried out at room temperature (RT) with mild shaking on orbital shaker. The plates were washed with PBS-Tween 20 (0.05%) and then blocked which was achieved by completely filling wells with blocking solution (1% BSA-PBS-Tween 20) for 2 hr. Plates were washed four times with PBS-Tween 20 and then 100 μl of hybridoma supernatant was added to different wells of ELISA plate. The plates were incubated for 2 hr and then washed four times with PBS-Tween 20. 100 μl of 1:1000 diluted rabbit anti-mouse IgG-peroxidase conjugate (Bangalore Genei) in 1% BSA-PBS-Tween 20 was added to individual well and plates incubated for 2 hr. The plates were washed five times with PBS-Tween 20. Subsequently 100 μl of substrate solution (4 mg o-phenylenediamine hydrochloride dissolved in 10 ml of 50 mM sodium citrate buffer pH 5.0 containing 0.01% H₂O₂) was added to individual wells. After 30 min., the reaction was terminated by addition of 100 μl 4N H₂SO₄ to each well. Absorbance was recorded at 420 nm in ELISA plate reader (Flow Lab, USA).

**Isotyping**—Classes and subclasses of antibodies from culture supernatant were determined by using mouse monoclonal sub-isotyping kit from Hyclone laboratories, USA and instructions given by manufacturer were followed.

**Dot-Blot**—Dot-blot was done on nitro cellulose (NC) membrane (Costar, USA). 1 μl of protein hormone dissolved in PBS was applied to glossy side of NC membrane. All incubations were carried out at RT with mild shaking on orbital shaker. After air drying, NC membrane was placed in blocking solution (3% BSA-PBS-0.05% Tween 20) for 1 hr. The membrane was washed once with PBS-Tween 20 and subsequently incubated with either 1:1 diluted hybridoma supernatant with 10 mM Tris-HCl, pH 8.0 containing 150 mM NaCl and 0.05% Tween 20 (TBST) or diluted ascitic fluid (1:500 in TBST) for 1 hr. Membrane was washed with PBS-Tween 20 (3 x 10 min) and then incubated with rabbit anti-mouse IgG-peroxidase conjugate (1:500 diluted in 1% BSA-PBS-0.05% Tween 20) for 2 hr. NC membranes were washed first with PBS-Tween 20 (3 x 10 min) and then with PBS (1 x 10 min.). Colour was developed by incubating membrane in substrate solution (6mg diaminobenzidine dissolved in 10 ml of 50 mM Tris-HCl, pH 7.6 containing 10 μl of 30% H₂O₂) till brown spots appeared on the membranes. Reaction was terminated by washing membranes with PBS. Membranes were then air-dried and photographed.

**Western-blot**—SDS-PAGE in slab gels was carried out as per the method of Laemmli. Discontinuous gel was prepared for mini dual vertical slab gel unit (M/s Bangalore Genei). 4% stacking gel was layered over 15% separating gel. Mw-markers-sample and bGH was prepared by boiling them for 5 min. in sample buffer (2% SDS, 5% β-mercaptoethanol and 10% glycerol). Other hormone and also bGH samples were prepared in sample buffer lacking β-mercaptoethanol (B-ME) without boiling. After electrophoresis, proteins from gel were transferred to NC membrane using mini trans-blot assembly from Bio-rad USA. The portion of membrane containing Mw markers was cut and stained with Ponceau S. The remaining portion of NC membrane was blocked with BSA-PBS Tween 20 and separated protein hormones were visualised as per the method described for dot-blot.

**Results**

From three fusion experiments, 188 wells out of 266 wells from 24-wells plates showed growth of fused cells corresponding to 70.7% fusion efficiency.
Screening of hybridoma supernatant showed that initially 26 out of 188 hybrids were tested positive against bGH in ELISA. Some of these hybrids grew poorly or lost the ability to secrete antibodies on subsequent culturing. Nine hybrids tested positive on sub-culturing were cloned. It was observed that cloning of five hybrids did not result in production of cloned hybridoma producing antibodies against bGH. However, cloning of four hybrids resulted in 11 cloned cells producing anti-bGH antibodies (Table 1).

The class or sub-class of antibodies produced by above hybridomas is shown in Table 1. The antibodies belonged to IgG1, IgG2b, and IgM class/subclass. The relative signal in ELISA as an index for production of anti-bGH-antibodies is also shown in Table 1. The signal in ELISA in supernatant of hybridomas is rather high indicating high level of antibody production. Since cloning of fused cells obtained from one particular well of 24-wells plate resulted in cloned cells producing antibodies of same class or subclass, it is likely that these could be identical clones. This allowed to select VIA6E8, VIB3C9B8, VIC1F9 and VIIB2E11C9 hybridomas for studying cross-reaction. The result of cross reaction of VIA6E8, VIB3C9B8, VIC1F9 and VIIB2E11C9 MAbs are shown in dot blot (Fig. 1) and Table 2. Two MAbs namely VIA6E8 and VIIB2E11C9 did not cross-react with ovine prolactin.

### Table 1—Class or subclass and relative signals of antibody produced by hybridomas

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<th>Name of hybridomas</th>
<th>Class/Subclass</th>
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<td>VIA6D4</td>
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<td>VIIB2D8B5</td>
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![Fig. 1—Detection of cross-reaction of MAbs using dot-blot. 2 μg of each hormone was spotted on nitrocellulose strips and blots were developed using different MAbs](image-url)
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(oPRL), ovine leutinizing hormone (oLH) and porcine follicle stimulating hormone (pFSH). MAb VIB3C9B8 cross-reacted with oPRL and oLH but not with pFSH. VIC1F9 antibody cross-reacted with oPRL and not with oLH and pFSH. None of the MAbs reacted with bGH when its disulfide bonds were reduced with B-ME. The possibility of minor contamination of growth hormone (GH) in oPRL and oLH preparations cannot be ruled out and low level of GH can be detected in dot-blot and then net result would be false cross-reaction. Thus, cross-reaction of VIB3C9B8 and VIC1F9 MAbs were also studied in Western blot where protein hormones (including impurities if any) were separated on the basis of their molecular weights and results are shown in Figs 2 and 3. Both these antibodies did not show cross-reaction with pFSH in both dot-blot and Western-blot. Similarly, these antibodies did not react in dot-blot and Western blot with bGH when its disulphide bonds were reduced. Blots in lane 2 and lane 4 from both figures (Figs 2 and 3) indicated that antibodies VIB3C9B8 and VIC1F9 truly cross-reacted with oPRL. However, in Fig. 3, an additional blot at position corresponding to dimer of oPRL could be observed indicating that VIC1F9 antibody reacted with dimer of oPRL. A faint additional band (not visible in Fig. 2) at similar position was also observed with antibody VIB3C9B8. The antibody VIB3C9B8 exhibited cross-reaction with oLH in dot-blot but not in Western blot.

Discussion

Based on cross-reaction results, there appears to be at least three epitopes against which antibodies were generated. VIA6E8 and VIIB2E11C9 did not cross-react with oPRL, oLH and pFSH and perhaps these antibodies could be against identical epitope. VIB3C9B8 and VIC1F9 antibodies are against different epitopes as cross-reaction results were not identical in dot-blot (Fig. 1) for the all the hormones tested. All the four antibodies were against conformational epitopes as all failed to react with denatured bGH (Fig. 1). Conformational epitopes in bGH are also shown by other workers and our results support this view. It has been reported that bGH maintained a high degree of secondary structure without intra-chain disulfide bond. Perhaps, minor alterations in secondary structure may result in distortion in epitopes. On the other hand report also exists where it has been shown that, on reduction of

<table>
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<tr>
<th>Hybridoma</th>
<th>bGH</th>
<th>bGH*</th>
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<th>oLH</th>
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Table 2—Cross-reaction of MAbs in dot-blot

*Iodination grade
**Reduced

Fig. 2—Detection of cross-reaction of VIB3C9B8 using Western-blot. 5 μg of each hormone was separated on 15% SDS-PAGE and transferred to nitrocellulose membrane. Lane 1, Mw markers (97.4, 66, 45, 31 and 21.5 kD). Lane 2, bGH. Lane 3, reduced bGH. Lane 4, oPRL. Lane 5 oLH. Lane 6, pFSH

Fig. 3—Detection of cross-reaction of VIC1F9 using Western-blot. 5 μg of each hormone was separated on 15% SDS-PAGE and transferred to nitrocellulose membrane. Lane 1, Mw markers (97.4, 66, 45, 31 and 21.5 kD). Lane 2, bGH. Lane 3, reduced bGH. Lane 4, oPRL. Lane 5 oLH. Lane 6, pFSH
disulphide bond in bGH, antigenic determinants are not lost and perhaps, these could be sequential epitopes. VIB3C9B8 antibody cross-reacted with oLH in dot-blot but not in Western blot. This is not unlikely as oLH in Western blot during electrophoresis comes in contact with SDS which may change conformation of oLH.

References
8 Harlow E & Lane D. in Antibodies ; A laboratory manual (Cold Spring Harbor Laboratory, New York) 1988, 245.