Anti-idiotypic Antibodies against Bovine Growth Hormone

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ABSTRACT : Anti-antibodies against three mouse monoclonal antibodies viz. IIB5D6, VIA6E8 and VIC1F9 (specific to bovine growth hormone) in rabbits have been generated and characterized. Ammonium sulfate fractionated and affinity-purified monoclonal antibodies were used for producing anti-antibodies. The generated anti-antibodies were against common as well as uncommon antigenic determinants present in mouse monoclonal antibodies. The raised anti-antibodies replaced [I^{125}]bGH bound to goat liver microsomes indicating production of anti-idiotypic antibodies against bovine growth hormone. These antibodies can have profound implications *in vivo* in lactating bovines for enhancing milk yield. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 5 : 732-737)***

Key Words : Anti-idiotypic Antibodies, Bovine Growth Hormone, Bovine Somatotropin, Receptor Assay, Antibody Purification

INTRODUCTION

Growth hormone (GH) is a protein hormone produced in somatotrophs of pituitary gland (Wallis, 1988). It comprises of about 191 amino acids with a molecular weight of 22 kDa. The major immunologically active zones are confined to 87-124 and 125-149 regions (Ferrara et al., 1979). Somatogenic as well as lactogenic activities are present in bovine GH (bGH). There are atleast three different conformational epitopes in bovine growth hormone (bGH) (Kumar and Rajput, 1999). The first in action of GH is the binding of GH to its receptor present in cell membrane of target tissues followed by dimerization of receptor, a known feature in the action of type I cytokines (Wells and deVos, 1996; Sodhi and Rajput, 2001).

Jerne (1974) proposed anti-idiotypic network theory and since then several anti-idiotypic antibodies against hormones have been raised. These include thyroid stimulating hormone (Baker et al., 1984), luetinizing hormone (Sairam et al., 1992), prolactin (Amit et al., 1986), follicle stimulating hormone (Udupa and Sheth, 1987), oestrogen (Mor et al., 1992) and growth hormone (Gardner et al., 1990; Schalla et al., 1994). In such studies, first polyclonal antibodies were raised and these antibodies were used for anti-idiotypic response.

bGH is known to enhance milk production in lactating animals and therefore it is interesting to know whether anti-idiotypic antibodies against this molecule can lead to enhanced milk production. Contrary to expectations, active immunization of lactating cows with bGH polyclonal antibodies had no effect on milk yield (Schalla et al., 1994). Perhaps MAbs against bGH, when used as immunogen in lactating bovines can enhance milk yield. However, before this concept is finally tested in bovines or buffalo, the generation of anti-idiotypic antibodies must be established in laboratory animals. In the present investigation, results on generation of anti-idiotypic antibodies against bGH are presented.

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Received June 21, 2002; Accepted November 21, 2002

MATERIALS AND METHODS

Three hybridoma cell lines viz. IIB5D6, VIA6E8 and VIC1F9 generated earlier and producing IgM, IgG_1 and IgG_{2b} antibodies against bGH were used (Kumar and Rajput, 1999).

Culturing of hybridomas

Frozen cells were thawed at 37°C and routinely cultured in RPMI-1640 medium containing 15% FCS. Cell lines growing poorly after thawing were rescued by supplementing medium with OPI mix and if necessary feeder cells were also provided (Harlow and Lane, 1988). 100 X OPI-mix was prepared by dissolving 1.5 g oxaloacetate, 0.5 g sodium pyruvate and 2000 I.U. insulin in 100 ml water. Culture supernatants from growing hybridoma cell lines were collected and supplemented with sodium azide (0.05% final concentration) and 1 ml of 1M Tris (pH 8.0) for every 20 ml supernatant. The supernatant was stored at -20°C till further use.

ELISA

ELISA was used to ascertain production of MAbs by hybridoma cell lines and method described by Engvall and Perlman (1971) with some modifications was followed. Briefly, multiwell plates were coated with bGH (1 μg/100 μl PBS, pH 7.4/well) by overnight incubations at 4°C. All subsequent incubations were carried out at room temperature with mild shaking on orbital shaker. Plates were washed with PBS-Tween 20 (0.05%) and then blocked by completely filling wells with blocking solution (1% BSA-PBS-Tween 20) for 2 h. After four times washing with PBS-Tween20, 100 μl of hybridoma supernatant (1:1

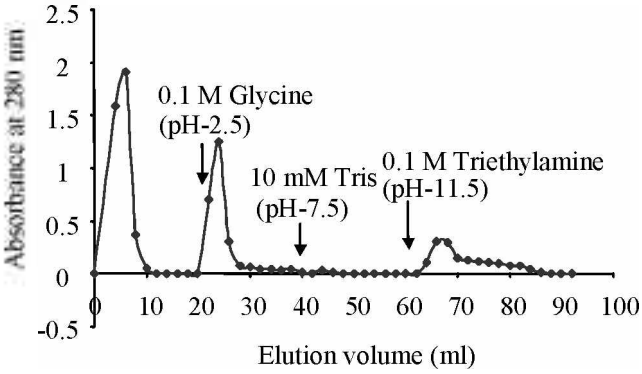

Figure 1. Affinity purification of mouse monoclonal antibodies using anti-mouse IgG agarose column. Ammonium sulfate fractionated proteins from hybridoma supernatants were loaded on affinity column. Bound proteins were eluted by 0.1 M glycine-HCl, pH 2.5 and 0.1M triethylamine, pH 11.5. Absorbance was recorded at 280 nm.

diluted with PBS) were added to different wells of ELISA plate. For negative and positive controls, 100 µl RPMI-1640 (15% FCS), 100 µl of mouse pre-immune (1:3,000 diluted with PBS) and mouse immune (1:3,000 diluted with PBS) were added. The plate was incubated for 2 h and then washed four times with PBS-Tween 20. 100 µl of 1:1,000 diluted rabbit anti-mouse IgG peroxidase conjugate (Bangalore, Genei) in 1% BSA-PBS-Tween 20 was added to individual well and the plate was incubated for 2 h. The plate was 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% H_2O_2) was added to individual well. After 30 min., the reaction was terminated by addition of 100 µl 4N H_2SO_4 to each well. Absorbance was recorded at 490 nm in ELISA plate reader (Toyo, Japan).

Purification of monoclonal antibodies

Antibodies were purified using ammonium sulfate precipitation and affinity chromatography techniques (Harlow and Lane, 1988).

Ammonium sulfate precipitation : To 100 ml tissue culture supernatants collected from hybridomas IIB5D6, VIA6E8 and VIC1F9, 31.3 g ammonium sulfate (providing 50% final saturation) was slowly added with constant stirring. The resultant precipitate was kept overnight at refrigerated temperature and centrifuged at 10,000×g for 20 min at 5°C. The pellet so obtained was dissolved in 10 ml of 10 mM Tris-HCl, pH 7.5 and dialyzed against 10 mM Tris-HCl, pH 7.5 overnight at 5°C with three changes. The dialyzed solution was centrifuged (10,000×g, 20 min) to remove any insoluble material. These antibody solutions were stored at -20°C after addition of sodium azide (0.05% final concentration).

Immunoaffinity purification of antibodies : Ammonium sulfate precipitated antibodies were further purified on goat anti-mouse immunoglobulin-agarose affinity column. These antibodies were loaded to affinity column (1.3×2 cm) equilibrated with 10 mM Tris-HCl buffer pH 7.5 by passing them thrice to the column to ensure binding of antibodies to the column. The unbound proteins were then removed by washing the column with 20 ml 10 mM Tris-HCl, pH 7.5. The bound proteins were then eluted by 100 mM glycine-HCl, pH 2.5. The column was again washed with 10 mM Tris-HCl, pH 7.5. Antibodies, which could not be eluted with glycine-HCl, were eluted by application of 0.1 M triethylamine buffer pH 11.5. Two ml fractions at a flow rate of 20 ml/h were collected. Fractions eluted with glycine-HCl and triethylamine were collected in tubes containing 200 µl 1 M Tris, pH 8.0. The eluted fractions were immediately mixed to bring the pH of eluted antibody to neutrality. Fractions eluted with glycine-HCl (elution volume between 22 to 26 ml, Figure.1) and triethylamine (elution volume between 64 to 68 ml, Figure.1) were pooled. These pooled fractions were concentrated by precipitating antibodies with ammonium sulfate (50% final saturation). The precipitate was dissolved in 10 mM Tris-HCl, pH 7.5 and dialyzed against the same buffer. These antibodies were either stored without sodium azide (for immunization of rabbits) or with 0.05% sodium azide (for hormone-receptor assay) at -20°C till further use.

Estimation of protein

The dye binding method of Bradford (1976) was used for estimation of protein.

SDS-PAGE

SDS-PAGE was carried out as per method of Laemmli (1970). Protein bands were visualized either by coomassie brilliant blue or ammonical silver stain (Harlow and Lane, 1988).

Immunization of rabbits

Each of the three MAbs viz. IIB5D6, VIA6E8 and VIC1F9 was administered to two rabbits (at least 2 months old). Primary injection (500 µg Mab emulsified with FCA) and three boosters (500 µg Mab in FIA) were given intramuscularly in hind legs. All immunizations were carried out after a gap of 21 days from each other. Ten days after last booster dose, blood was collected from ear vein and serum separated. Sodium azide (0.05%) supplemented serum was stored at -20°C till further use. For hormone receptor assay, antibodies from sera were precipitated with 50% saturated ammonium sulfate, dialyzed against PBS containing 0.05% sodium azide and then stored in small aliquots at -20°C.

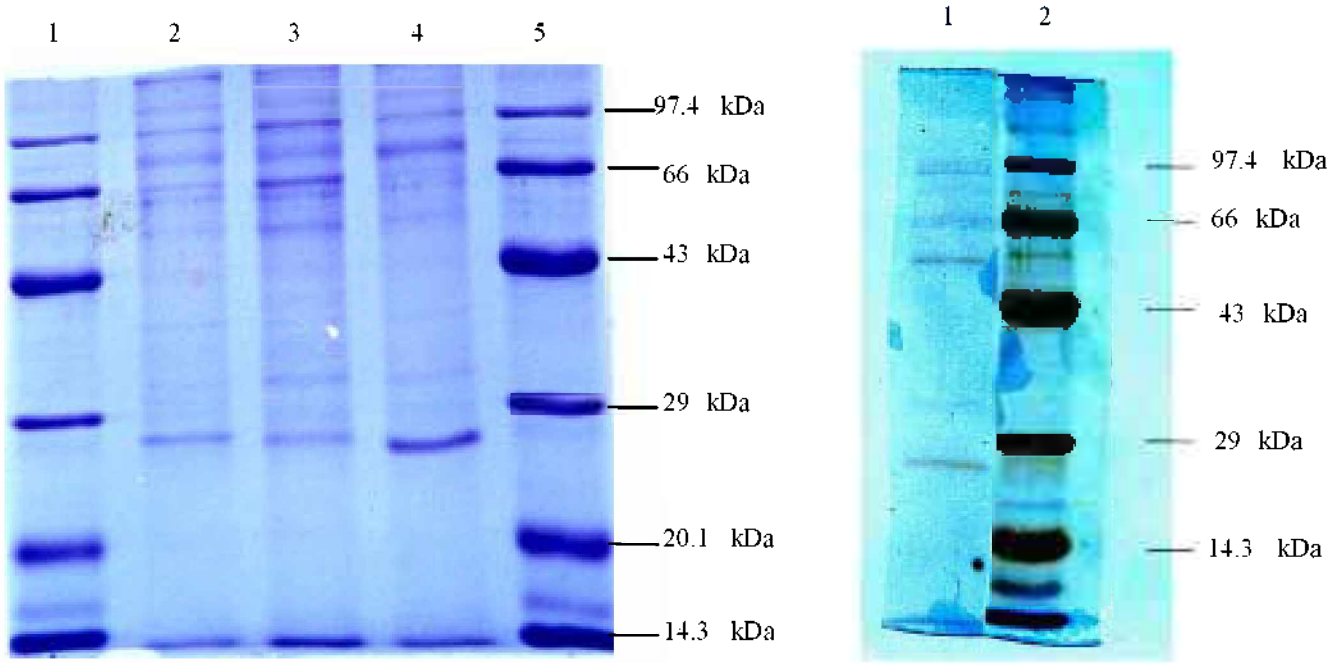

Figure 2. (a): SDS-PAGE profile of monoclonal antibodies (Mabs). Mabs purified by ammonium sulfate fractionation and affinity chromatography were separated on 12.5% acrylamide gel. Protein bands were visualized by coomassie brilliant blue R. Lane 1 and 5, molecular weight markers (30 µg); Lane 2, II B5D6 antibody (5 µg); Lane 3, VI C1F9 antibody (5 µg); Lane 4, VI A6E8 antibody (7 µg)
(b): SDS-PAGE pattern of VIA6E8 .VIA6E8 antibody purified by ammonium sulfate fractionation, affinity chromatography and rechromatography on affinity column. Protein bands were visualized by silver staining. Lane 1, VIA6E8 antibody (1 µg); Lane 2, Molecular weight markers (10 µg).

Immunodiffusion

The presence of antibodies in sera obtained from immunized rabbits was tested in immunodiffusion as described by Nilsson (1984). The precipitation lines were visualized by coomassie brilliant blue R (Nilsson, 1984).

Hormone – receptor assay

Labelling of bGH : bGH was labelled with iodine by the method of Fraker and Speck (1978) as described by Cadman and Wallis (1981) with some modifications. Iodogen coated eppendorf tubes (0.5 ml) were prepared as follows. 2 µg iodogen (dissolved in 40 µl chloroform) was added to eppendorf tube. The tube was held in palm and continuously rotated in horizontal position to enable uniform coating of iodogen at maximum surface area. bGH (20 µg/35 µl 0.5 MPO_4, pH 7.4) was added to iodogen–coated eppendorf tube. Immediately, 5 µl NaI^{125} (500 µCi) was added. Reaction was stopped by adding 200 µl 25 mM Tris -HCl, pH 7.5 containing 0.05% BSA and 0.05% sodium azide (TBA-buffer). Contents were mixed and loaded on sephadex G-50 column equilibrated with TBA-buffer. Fractions containing iodine bound to bGH were pooled and appropriately diluted with TBA-buffer to give 150.000 cpm per 100µl.

Microsome preparation : Liver microsomes were prepared as per the method of Haro et al. (1984) with some modifications. Liver from freshly slaughtered goat was collected, immersed in cold saline and brought to the laboratory. A piece (~20 g) of liver was placed in the petri dish and rinsed several times with cold saline. About 50 ml chilled Tris-buffer (25 mM), pH 7.0 containing 10 mM EDTA, 10 mM EGTA, 300 mM sucrose, 0.6 mM PMSF, 1 µM leupeptin and 1 µM pepstatin A was added, tissue was cut into small pieces and homogenized for one minute by giving five strokes. Homogenized tissue was filtered through eight layers of cheese cloth. The filtrate was centrifuged at 10,000×g for 15 min. at 5°C. Pellet was discarded and supernatant was ultracentrifuged (100,000×g, 90 min., 5°C). The pellet was resuspended in 25 mM Tris, pH 7.6 containing 10 mM EDTA, 10 mM EGTA, 0.6 mM PMSF, 1 µM leupeptin and 1 µM pepstatin A (Buffer A). Suspended pellet was uniformly mixed by homogenizing it for one minute by giving five strokes. Microsome preparation was diluted with buffer A to give protein concentration of 3 mg/ml and stored in small aliquots at -70°C.

Table 2. Replacement of bound [I^{125}] bGH to goat liver microsomes by bovine growth hormone and different anti-antibodies

Additives	cpm±standard eviation	% Substitution of control
Control	2,9747±1,080	0
bGH (10μg)	2,0971±677	30
VIA6E8 (1μg)	2,0970±1,527	30
VIA6E8 (8μg)	1,9265±980	35
VIA6E8 (50μg)	1,8152±1,787	39
IIB5D6 (1μg)	2,0563±1,972	31
IIB5D6 (8μg)	1,9196±944	35
IIB5D6 (50μg)	1,5814±529	47
VIC1F9 (1μg)	2,1039±1,177	29
VIC1F9 (8μg)	2,1876±488	26
VIC1F9 (50μg)	2,1866±265	26

* Figures in parenthesis indicate amount of bGH or anti-antibodies present in 500 μl reaction volume.

Assay : Hormone –receptor assay was performed as per the method of Cadman and Wallis (1981) with some modifications. This assay was carried out in 2 ml microcentrifuge tubes. Reaction mixture (500 μl) contained 100 μl microsomes (300 μg protein), 100 μl [I^{125}] bGH (1,50,000 cpm) and appropriate volume of assay buffer (25 mM Tris, pH 7.6 containing 10 mM EDTA, 10 mM EGTA, 0.6 mM PMSF, 1 μM leupeptin, 1 μM pepstatin A, 0.1% BSA and 0.05 % Na-azide). The replacement of bound I^{125} bGH with cold hormone or rabbit anti- (mouse Mabs) antibodies was studied by adding 10 μg bGH or 1.8 and 50 μg antibodies dissolved in 100 μl assay buffer.

The reaction mixture was incubated at 25°C in water bath for 5 h. Then, 1.5 ml 25 mM chilled Na-acetate, pH 5.4 was added to stop the reaction. After 10 min, tubes were centrifuged at 4,000×g for 25 min at 5°C. Supernatant was carefully removed. Traces of supernatant attached to walls were removed with help of filter paper without disturbing the pellet. Counts in pellet were recorded in γ-counter.

RESULTS AND DISCUSSION

Purification of antibodies

Culture supernatants from hybridoma cell lines IIB5D6, VIA6E8 and VIC1F9 were the source of Mabs against bGH. These cell lines were secreting Mabs as clear from signal in ELISA (Table 1). The culture supernatants from hybridoma cells grown *in vitro* contain antibodies of mouse origin and immunoglobulins of bovine origin. Ammonium sulfate fractionation separates immunoglobulins from other serum proteins present in culture supernatant. Subsequent purification of immunoglobulins on anti-mouse IgG antibodies affinity column will separate mouse immunoglobulins from bovine immunoglobulins and elution profile is shown in Figure1. The SDS-PAGE pattern of IIB5D6, VIA6E8 and VIC1F9 (Figure. 2a) indicated that in all antibody preparations, a protein band corresponding to light chain (25 kDa) was distinct. In case of IIB5D6 (lane 2), a intense band corresponding to μ chain was noticed. Minor contaminants of higher or few lower molecules weight proteins than μ chain were also seen but overall preparation of IIB5D6 appeared to be reasonably pure (>80%). Antibodies VIC1F9 (lane 3) and VIA6E8 (lane 4) showed two doublet bands (~60 kDa) and one major band of approximately 75 kDa. It is expected that intensity of heavy chain should be atleast twice of light chain but this was not found with any of the three antibodies. There are few possibilities. Heavy chain is not completely dissociated from light chain and this can result in bands of higher molecular weight. Second possibility is that heavy chain is degraded by proteolytic enzyme since presence of one such enzyme viz. cathepsin D has been established in hybridoma culture supernatants (serum-free) collected from dead or lyzed cells (Erp et al., 1991a). Possibility also exists that clone specific proteolytic enzyme is present (Erp et al., 1991b). Other workers also observed degradation of heavy chain (Chen et al., 1984) and some workers referred as extra band as slow moving light chain (Lee, 1987). The present observation and observations of few other workers suggest the need for working out SDS-PAGE protocol for immunoglobulins where artifacts can be eliminated or minimized. Because of possible artifacts in movement of heavy chain, it is difficult to predict the purity of preparations. Nevertheless, it is also very unlikely that the preparations were without any contaminants. Emtner et al. (1989) could obtain purity greater than 75% using protein A affinity column and in present case also electrophoretically homogeneous preparation could not be obtained.

Table 1. Relative antibody signals produced by hybridomas

Hybridoma/controls	Absorbance, 490nm
Medium (RPMI)	0.020
IIB5D6 (IgM)	0.400
VIA6E8 (IgG_1)	0.300
VIC1F9 (IgG_{2b})	0.347
Preimmune serum	0.000
Immune serum	0.361

Rechromatography of VIA6E8 on antimouse immunoglobulin was done in order to improve purity of preparation and SDS-PAGE pattern is shown in Fig. 2b. There is improvement in purity but bands higher or lower than γ-chain could also be detected. In general, from 100 ml hybridoma culture supernatant, about 5 to 9 mg of antibodies were obtained.

Production of anti- (bGH antibodies) antibodies in rabbits

Three MAbs viz. VIC1F9, VIA6E8 and IIB5D6 were used for raising antibodies in rabbits and the presence of

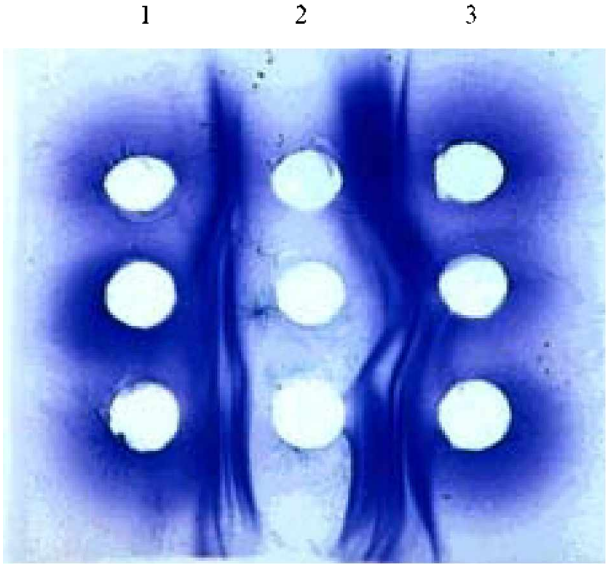

Figure 3. Testing presence of anti-antibodies using immunodiffusion technique.For each antibody, two rabbits were immunized.Column1 and column 3 contained serum from different rabbits immunized with Mabs.Column 2 contained Mabs.Row 1 contained VIC1F9 antibody and antisera against it.Row 2 contained VIA6E8 antibody and antisera against it.Row 3 contained IIB5D6 antibody and antisera against it.

raised antibodies was tested in sera by immunodiffusion method. The precipitation lines were observed with all the three MAbs with corresponding antisera (Fig. 3). Sera obtained from two different rabbits immunized with same MAb did not give same number of precipitation lines. For example, antiserum from one rabbit (bottom row, right column) gave five precipitation lines against IIB5D6 antibody whereas other rabbit (bottom row, first column) produced only four precipitation lines. Similarly, one extra precipitation line is observed between VIA6E8 antibody and antiserum (middle row, first column) as compared to immune serum collected from other rabbit (middle row, right column). This is even true for the MAb VIC1F9 where one of the rabbit serum (top row, right column) produced one extra precipitation line as compared to other serum (top row, first column). One interesting observation in the design of immunodiffusion experiment is that some continuous precipitation lines all across the three MAbs and respective antisera are seen. These may be against common antigenic determinants present in all the three MAbs. Titre in serum from one rabbit (middle row, right column) appears to be low as precipitation lines are pushed away from central well (middle row, middle column) containing VIA6E8 antibody. These results clearly indicated that antibodies against all the three MAbs are generated in rabbits.

Anti-idiotypic antibodies against bGH

Rabbit anti- (mouse immunoglobulin) antibodies will be pool of antibodies generated against class specific determinants, common antigenic determinants to other classes, species-specific determinants, allotopes and iodiotopes. Amongst these antibodies, anti-idiotypic antibodies may bear internal image of original antigen. These anti-antibodies were tested for their ability to replace bound [I^{125}] bGH to goat liver microsomes and results are shown in Table 2.

Anti-VIC1F9 antibodies over the concentration range 1 μg to 50 μg/500 μl was in saturation range and showed replacement equivalent to replacement obtained with 10 μg cold hormone. On the other hand anti- VIA6E8 and IIB5D6 antibodies at 50 μg /500 μl concentration showed higher replacement as compared to 10 μg cold hormone. The variable response of these anti-antibodies could be due to (i) the relative abundance of anti-idiotypic antibodies in preparation and (ii) the possibility that anti-antibodies VIA6E8, IIB5D6 and VIC1F9 are generated against different epitopes of bGH whose contributions in binding of bGH to receptor are variable. 50-70% non specific binding of [I^{125}] bGH to goat liver microsomes was observed and this value appears to be high. The literature on this aspect is silent except one report in which specific binding in the range 55-75% with microsomes and 40-60% with hepatoma cell line have been shown (Elbashir et al., 1990).

These results demonstrated that all the three anti–antibodies were able to replace [I^{125}] bGH bound to goat liver microsomes suggesting that generated anti-antibodies against all the three Mabs viz IIB5D6, VIA6E8 and VIC1F9 bear internal images of bGH and qualify to be called anti-idiotypic antibodies.

ACKNOWLEDGEMENT

Authors are thankful to USDA for providing gift samples of bGH

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