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Original Research Article

Production of Monoclonal Antibodies a Thyroid Stimulating Hormone (TSH) using Mice as an Animal Model

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Abstract

carried out. Two inbred BALB/C mice were immunized with recombinant TSH injection and checked for immune response by ELISA. The TSH antibody response of both immnunized animals as tested in TSH-coated wells using goat anti-mice IgG-HRP for detection. The spleen of mouse No. 2 showed better antibody response and used for fusion of spleen with myeloma cell line ($SP^2/0$). After fusion, hybrid cell confluences were tested in antigen coated wells by antibody capture ELISA. Of 6 clones, three clones were chosen for expansion and further subcloning and limited dilutions were made. Based on ELISA response four subclone were chosen for P_6D_4 ($P_6D_4C_3$, $P_6D_4B_7$, P_6D_4 B_8, P_6D_4 G_8) and one subclone for test two clones ($P_4B_2H_3$ and $P_1A_{10}E_3$) and tested for their binding to other glycoprotein hormones (hCG) and its subunits. All of them showed high binding to alpha- hCG, then to TSH followed by beta hCG. In hCG coated wells, the antibody response was very low, suggesting some overlap in recognition of epitopes. So, these hybrid cells were subcloned further at least two times to ensure monoclonally of the cells. The antibody seemed to be mainly against alpha subunit of hCG assuming that it was directed at alpha subunit of TSH. A second fusion using TSH (recombinant) mouse was also carried out and a single clone P_4B_{12} was obtained, which was sub-cloned to get monoclonal antibody directed against single epitope. Comparatively to the previous fusion, binding was less in these clones, but directed at the same antigen. Antibody response of ascetic fluid of $P_1A_{10}E_3$ was tested at different dilutions of cells. This particular clone showed very high titre showing binding to alpha hCG, followed by TSH, β-TSH and minimum in hCG. The maximum protein was recorded in $P_4B_{12}B_9$ (1.869mg/ml) (ascetic fluid) and total protein (6.564mg) in $P_1A_{10}E_3$ clone (ascetic fluid). The maximum protein was recorded in $P_4B_2H_9D_9C_3$ (1.869mg/ml) and total Protein (6.678mg) in $P_1A_{10}E_3G_{12}$ clone (supernatant/culture medium). The present findings suggested that purification and characterization of the monoclonal antibody are needed for further use.

Keywords: *Antibody, Monoclonal, ELISA, TSH, hCG, Hormone, Mice.*

1. Introduction

Thyroid-stimulating hormone (TSH) is also known as thyrotropin, a glycoprotein hormone synthesized and secreted by thyrotrope cells in the anterior pituitary gland, which regulates the endocrine function of the thyroid gland. TSH is composed of two subunits i.e. alpha subunit and beta subunit where alpha subunit binds to beta subunit. The alpha subunit is almost identical to that of human chorionic gonadotropin (hCG), follicle-stimulating hormone (FSH), and luteinising hormone (LH). The beta subunit is unique to TSH and determines its receptor specificity. The determination of serum or plasma levels of thyroid stimulating hormone (TSH or thyrotropin) is recognized as a sensitive method in the diagnosis of primary and secondary hypothyroidism (Burger and Patel, 1977).

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TSH is secreted by the anterior lobe of the pituitary gland and induces the production and release of thyroxine and triiodothyronine from the thyroid gland (Ezrin, 1978). It is a glycoprotein with a molecular weight of approximately 28,000 daltons, consisting of two chemically different subunits, alpha and beta (Pierce, 1971). The thyrotropin receptor, also known as the thyroid-stimulating hormone receptor (TSHR), is the primary antigen of Graves disease. Stimulating TSHR antibodies are the cause of thyroid overstimulation and were originally called long-acting thyroid stimulators due to their prolonged action (Davies *et al*., 2002).

The beta chains are distinct but do contain regions with identical amino acid sequences. These regions of homology can cause considerable cross-reactivity with some polyclonal TSH antisera. The use of a monoclonal antibody in this TSH ELISA test eliminates such cross-reactivity, which could result in falsely elevated TSH values in either menopausal or pregnant females-a

population whose evaluation of thyroid status is clinically significant (Soos and Siddle, 1982; Wada *et al*, 1982; Snyder and Utiger, 1982). The levels of TSH and TSH-releasing hormone (TRH) are inversely related to the level of thyroid hormone. When there is a high level of thyroid hormone in the blood, less TRH is released by the hypothalamus, so less TSH is secreted by the pituitary. The opposite action will occur when there is decreased thyroid hormone in the blood. This process is known as a negative feedback mechanism and is responsible for maintaining the proper blood levels of these hormones (Berger *et al*. 1976; Utiger, 1978). Thyrotropin-stimulating hormone (TSH) is a noncovalently linked glycoprotein heterodimer and is part of a family of pituitary hormones containing a common alpha subunit and a unique beta subunit that confers specificity. Free alpha and beta subunits have essentially no biological activity. TSH is secreted from cells in the anterior pituitary and it is indispensable for the control of thyroid structure and metabolism. The quality of the target antigen is very important in order to generate a good antibody, in particular when binding to a conformational epitope is desired. Monoclonal antibodies are typically made by cell culture that involves fusing myeloma cells with mouse spleen cells immunized with the desired antigen (*Beck et al*., 2010). Keeping in view the above justification, studies on monoclonal antibody production against recombinant thyroid stimulating hormone (TSH) was carried out.

2. Materials and Methods

Reference standards of beta –TSH and TSH were procured from National Institute of Biological Standards and controls (U. K) and TSH-RP-2 obtained from National Institute of Arthritis and Metabolism Diseases, NIH, Bethesda, MD and made available by Hormones Research Foundation (HRF). hCG and FSH from NIBSC were also available with HFR. Myeloma cells ($SP^2/0$) were obtained from National Centre for Cell Science, Pune, India. Various media such as RPMI, FCS, HT and HAT were procured from HIMEDIA and Hybrimax.

2.1. Immunization of BALB/C mice

Recombinant TSH was checked for its biological activity in ELISA for TSH, before used for immunization. Primary injection of 10µg each was emulsified with saline and Freund's complete adjuvant in 1:1 (v/v) proportion made by mixing thoroughly using a 18'' Needle, subcutaneous injections were given over head, back and tail. This was followed by a 5µg injection prepared in saline: Freund's complete adjuvant 1:1 (v/v) and injected subcutaneously. After three injections, mice were bled from retro-orbital vein of the eye under mild anesthesia on the 8^{th} day after injection. Serum was prepared and tested using different dilutions (1:100,1:200 and 1:300) in TSH or α- hCG coated wells with anti-mouse IgG-HRP as lebel. Animal that showed the highest antibody titres more than 1:100 was selected. Two to three days prior to fusion, the mouse was injected with 10µg of TSH in 1ml saline intraperitonially.

2.2. Monoclonal antibody production

One vial of RPMI powder was dissolved in 500ml of sterile double distilled water. RPMI was rinsed with water, so that remnant of powder stuck to the wall of the contained could also be transferred and dissolved. Sodium bicarbonate was dissolved properly and adjusted pH 7.2. The medium, thus prepared was filtered through a sterile 0.22µm membrane filter. Aliquots of 50ml (without antibiotic) were stored in glass bottles at -20^0C.

2.3. Growth of Myeloma cell lines

Myeloma cells ($SP^2/0$) were grown in RPMI 1600, supplemented with 10% FCS having antibiotic antimycotic solution (1.0ml/100). The flask were inspected for live dividing cells everyday and cells were counted and maintained at 37^0C in a humid atmosphere 5% CO_2 and 95% O_2. The cells were scrapped and centrifuged at 800rpm for 10 minutes and re-suspended in fresh medium. The cells were allowed to grow till the growth reached to $5x10^6$ cell/ml in the flask. The myeloma cells were preserved/ maintained at -70^0C for 24 hours and later stored in liquid nitrogen for long term storage.

2.4. Fusion Protocol

2.4.1. Feeder layers of Spleen cells

This was done by plating normal mouse spleen as feeder layes one day prior to fusion. Connective tissues were removed and changed to another Petri plates with wash medium. Wire mess and forceps were heated over gas burner and wire mess was placed in the medium. Spleen was removed from BALB/C mouse under aseptic conditions and placed in a Petri dish having ash medium. Wire mess and forceps were heated over gas burner and wire mesh was placed in the medium. Spleen was placed on the wire mess and cells were treated with blunt forceps and rubbed against the mess. The medium having cells was transferred to 15 ml sterile centrifuges tubes, leaving out layer chunks of tissues. It was centrifuged at 500g for 10 min and pellets was suspended in 5ml of cold 0.17M NH_4Cl solution and kept over ice for 10min. To this 5ml of 10% FCS-medium was added and centrifuged at 1000rpm for 5min. The pellet was re-suspended in 10 ml of 20% FCS-medium. Cells were counted in a hemocytometer using 0.4% trypan blue solution. Around 25,000 – 50,000 cells per 50 micro-liter of medium were placed in each well of 8x96 wells, after diluting the cells to required volume with FCS medium. The plates were kept at 37^0C in a CO_2 incubator having supply of 5% CO_2.

2.4.2. Fusion of Immunized Mouse spleen cell with $SP^2/0$ Cells

On second day, mouse injected with recombinant TSH (RTSH) was bled from retro-orbital plexus of eye vein under anesthesia. Serum was prepared from blood and used as positive control. Spleen was collected from mice. After first centrifugation, the cells were suspended in 5 ml of 10% FCS-medium. Centrifuged again at 1600 rpm for 10min and supernatant was discarded. The pellet was re-suspended in 5ml of serum free medium. An aliquot of 50µl was taken and 450µl of cold 0.17M NH4Cl was added and kept for min over ice. Cells were counted using trypan blue exclusion method in a hemocytometer.

Myeloma cells ($SP^2/0$) as already maintained in a log phase were scraped from culture flasks and centrifuges at 1000 rpm for 10 min. The pellet was suspecded in 10ml washing medium and cells were counted using hemocytometer. Myeloma cells and spleen cells were combined in the ration of 1:6 proportion in a 50ml centrifuge tube and centrifuged at 1000rpm for 10 min. The combined cell pellet was again washed with 40ml wash medium and centrifuges again and ensured that the medium was completely removed.

Both 10% FCS-medium and 20%FCS-medium were kept at 37^0C, before starting the fusion procedure. Water was heated in a beaker to 40^0C. After ensured that the medium was completely removed, the centrifuge tube was tapped from the bottom and sides to dislodge the pellets. The tube was kept in hot water (heated to 40^0C). Poly ethylene glycol was added to the cells slowly over a minute, while swirling the contents gently at 37^0C. This was continued for another minute and in the third minute 1ml of medium without serum was slowly added, while during the 4th minute. In the 5th minute, 5ml of minutes was added. During the 6th minute, the medium was made up to 40ml. The cells were pelleted at 800rpm for 5min. The supernatant was discarded and the cells were re-suspended in 3ml of 20% FCS medium. Using a Pasteur pipette, the clumps were broken carefully. It was then diluted to required volume, so that 100µl/well could be added to 8x96 well plates having feeder layer. The plates were left at 37^0C in a CO_2 incubator and left undisturbed for 24hours.

On the second day, 175µl of HAT medium was added to each well. After six days 200 µl was removed from each well and supplemented with 200 µl/well HAT-medium. While aspirating medium from the wells of the plates, care was taken not to disturb the cells at the bottom. Screening the presence of hybrid cells was started from 9th day and at regular interval.

2.5. Screening assay for monoclonal antibody activity

Twelve to 15 days after fusion, culture supernatants showing the presence of hybrid cells were tested for the presence of antibody by antibody capture assay. Coating the microtiter plate was done and ELISA was performed to determine the concentration of antibody.

2.6. Development of hybridoma cell line

Those cells showing presence of antibody and in confluence were scraped and expanded to 24-well plates having 0.5ml of RPMI containing HT (1X) in 20% FCS medium. The rest of the cells were kept in original 96 well plate for 'back up' culture. This served as a precaustionary measure in case contamination occurred in 24 well plate, then cells could observed every two days for their growth and fresh medium containing HT was added to a final volume of 2.0ml. The hybrids growing as 'bake up' in 96 well plates were also taken in another well of a 24 well plate in 'duplicate' well. Once a positive well showed growth reaching (1/3-1/2) confluence in 24 well plate, the supernatant were again tested for desired antibody by ELISA. Positive cultures were expanded in 6 well plates in complete medium comparising 15% FCS, with repeated determination for the presence of the antibody. Hybrid cells secreting the desired antibody were also frozen by pooling the contents of two confluent cups of 24 well plates. The cell clones were selected for sub-culturing. The stable hybrid showing good density 5-10million and injected in pristane treated mice to get ascetic fluid.

2.7. Ascites fluids production

For large scale antibody production ascetic fluid production was done BALB/C mice were primed with 500 µl of pristane intraperotonially at least one week prior to injecting the cells. An amount of approximately $5x10^6$ cells were spun, washed once in PBS, and resuspecded into 500 microliter of PBS. The cells were then injected (500 microliter of cells) per mouse intraperitoneally. After 7-14days the ascites should begin to form. The mice were tapped sometimes as many as 3 times in a 7 days period, resulting in enough ascites per mouse. The ascites fluid was centrifuge a 3000rpm for ten minutes to remove the blood cells and stored at -20^0C until it was purified.

3. Results

3.1. Immunization and immune response

For the production of monoclonal antibody, two inbred BALB/C mice were immunized with recombinant TSH injection and checked for immune response by ELISA using serum prepared from blood taken from retro-orbital vein of the eye. The TSH antibody response of both immnunized animals as tested in TSH-coated wells using goat anti-mice IgG-HRP for detection as shown in Table 1. The spleen of mouse No. 2 showed better antibody response and used for fusion of spleen with myeloma cell line $SP^2/0$.

Table 1 TSH antibody response of immnunized animals tested in TSH-coated wells using goat anti-mice IgG-HRP

Animal No.	Dilution of Serum	OD at 450nm	Animal No.	Dilution of Serum	OD at 450nm
1	1:50	1.161	2	1:50	1.740
	1:100	1.289		1:100	1.769
	1:200	1.174		1:200	1.762
	1:400	1.128		1:400	1.610

3.2. Fusion

After fusion, 100µl of fused cells were added to each well having feeder layer of spleen cells and HAT medium was added after 24 hr., hybrids formed were selected in HAT medium and replaced after 5 days. After 12-15 days of fusion the plates were further screened using culture supernatant of well shoing hybrid cell confluences. These were tested in antigen coated wells by antibody capture ELISA, Of the 142 wells showing hybrid, 8 clones showed positive response. The following hybrids were chosen for further expansion and cloning (Table 2).

Table 2 Antibody response of culture supernatant on 10th day against TSH

Culture supernatant of clones	TSH/α-HCG
Medium	0.389
Positive Serum (1:50) from Mice	3.367
P_1G_6	0.571
P_4B_2	0.802
P_5E_{12}	0.560
P_6A_1	0.668
P_7A_4	0.733
P_6D_4	1.575
P_1A_{10}	0.629
P_4D_3	0.534

Of 8 clones, 6 showed strong positive signals were picked and further transferred to 24 well plate for expansion. Of 6 clones, three clones were chosen for expansion and further subcloning and limited dilutions were made.

Using these dilutions, cells were grown and many wells found to show positive antibody by binding to alpha hCG coated plates. Based on ELISA response four subclone were chosen for P_6D_4 ($P_6D_4C_3$, $P_6D_4B_7$, $P_6D_4B_8$, $P_6D_4G_8$) and one subclone for test two clones ($P_4B_2H_3$ and $P_1A_{10}E_3$) and tested for their binding to other glycoprotein hormones (hCG) and its subunits. The culture supernatant of these clones was found to be working from direct supernatant to a high titre of 1:12000 dilution as shown in Table 3.

Table 3 Antibody response of culture supernatants in TSH, beta hCG and hCG coated wells (1:2000 dilution)

Clone	TSH (10ng)	Beta-hCG (1µg)	Alpha-hCG (1µg)	hCG (1µg)
	Absorbance at 450nm			
$P_6D_4C_3$	0.923	0.588	1.453	0.306
$P_6D_4B_7$	1.012	0.928	2.402	0.207
$P_6D_4B_8$	0.552	1.359	1.795	0.158
$P_6D_4G_8$	0.960	0.366	1.142	-
$P_4B_2H_3$	0.825	0.787	1.941	0.274
$P_1A_{10}E_3$	1.606	1.012	2.217	0.517

All of them showed high binding to alpha- hCG, then to TSH followed by beta hCG. In hCG coated wells, the antibody response was very low, suggesting some overlap in recognition of epitopes. So these hybrid cells were subclones further at least two times to ensure monoclonally of the cells.

Table 4 Specific antibody response of culture supernatant after subcloning the initial hybrid cells

Clone	TSH (10ng)	Beta-hCG (1µg)	Alpha-hCG (1µg)	hCG (1µg)
	Absorbance at 450nm			
$P_6D_4B_7G_9$	0.641	0.013	2.191	0.221
$P_6D_4B_7H_2$	1.000	0.204	1.725	0.552
$P_6D_4B_7H_2H_6$	1.552	0.601	2.486	1.239
$P_6D_4G_8H_9$	0.967	0.220	0.265	-
$P_6D_4G_8H_9G_{12}$	0.815	0.201	1.512	0.639
$P_6D_4G_8H_9G_{12}C_{12}$	2.317	0.631	2.521	1.994
$P_6B_4H_9D_9$	1.020	0.397	2.997	0.959
$P_6B_4H_9D_9C_3$	2.948	0.903	2.483	2.915
$P_1A_{10}E_3G_{12}$	0.758	0.189	2.674	0.595

The antibody seemed to be mainly against alpha subunit of hCG assuming that it was directed at alpha subunit of TSH. A second fusion using TSH (recombinant) mouse was also carried out and a single clone P4B12 was obtained, which was sub-cloned to get monoclonal antibody directed against single epitope. These were tested in coated wells as shown in the Table 5. Comparatively to the previous fusion, binding was less in these clones, but directed at the same antigen.

Table 5 Second fusion and sub-cloning

Clone	TSH (10ng)	Beta-hCG (1µg)	Alpha-hCG (1µg)	hCG (1µg)
	Absorbance at 450nm			
$P_6D_4B_7G_9$	0.641	0.013	2.191	0.221
$P_6D_4B_7H_2$	1.000	0.204	1.725	0.552
$P_6D_4B_7H_2H_6$	1.552	0.601	2.486	1.239
$P_6D_4G_8H_9$	0.967	0.220	0.265	-

3.3. Production of Antibody

Different clones were injected into BALB/C mice treated with pristine and six ascetic fluid samples were collected. Antibody response of ascetic fluid of $P_1A_{10}E_3$ was tested at different dilutions of cells (Table 6).

Table 6. Titre of crude ascites fluid of P1A10E3 clone

Dilution/ titre	TSH (10ng)	Beta-hCG (1µg)	Alpha-hCG (1µg)	hCG (1µg)
	Absorbance at 450nm			
Ascitic 1:2000	1.385	0.961	3.356	0.659
1:4000	1.268	1.109	3.356	0.627
1:8000	1.082	1.003	3.356	0.627
1:12000	1.137	1.051	3.356	0.458
1:16000	0.922	0.896	3.356	0.454
1:20000	0.874	1.014	3.234	0.398

This particular clone showed very high titer showing binding to alpha hCG, followed by TSH, Beta TSH and hCG. Protein content of IgG fractions of ascetic fluid and culture supernatant that were available for further work.

The protein amount present in these purified IgG was calculated by spectrophotometric readings at 280nm and 260nm (Table 7).

Table 7 Protein content of IgG fractions of different clones

Clone (supernatant or ascetic fluid)	Protein (mg/ml)	Total Vol. (in ml)	Total Protein (mg)
$P_4B_{12}D_9$ (ascetic fluid)	1.736	3.0	5.208
$P_1A_{10}E_3$ (ascetic fluid)	1.094	6.0	6.564
$P_4B_{12}B_9$ (ascetic fluid)	1.869	2.0	3.738
$P_4B_{12}G_9$ (ascetic fluid)	2.28	1.0	2.28
$P_6D_4G_8H_9G_{12}C_{12}$ (Culture medium)	1.118	3.5	3.913
$P_1A_{10}E_3G_{12}$ (Culture medium)	1.694	4.0	6.678
$P_4B_2H_9D_9C_3$(Culture medium)	1.199	3.0	3.597
$P_6D_4B_7H_2H_6$(Culture medium)	1.913	3.0	5.736
$P_4B_{12}B_9$(Culture medium)	1.326	3.5	4.641

4. Discussion

In the present study, two BALB/C mice were immunized for the production of monoclonal antibody against recombinant TSH (RC-TSH). After determining the antibody response the spleen cells were fused with $SP^2/0$ myeloma cells in the 6:1. From two separate fusion, several hybrids with positive response were obtained. Of which 3 hybrids from the first fusion, one from second fusion were chosen for expansion and subcloning. This resulted in clones showing the high antibody response in the culture supernatant against α-hCG (here α-hCG was used in place of α-TSH) and TSH and to some extent also against β-hCG . Then the clones were further subcloned to get hybrid of monoclonal origin and possibly directed against single epitope. All of them showed similar reactivity patterns including ascetic fluid produced for some of the clones in pristane treated BALB/C mice. The total protein (mg/ml) was also determined in the present endeavour.

The objective was to develop mABs against TSH that can uses in ELISA protocol. Production of monoclonal antibodies against different epitopes of TSH required for use in the assay for in vitro quantitative determination of TSH in human serum was a major necessity. (Gow *et al*., 1985; Hayashizaki *et al*.,1985). Attempts were made to develop monoclonal antibody following the procedures described by Kohlar and Milstein (1981). Kashiwal *et al*., (1991) reported that recombinant human thyrotropin (RC-TSH) produced in Chinese hamster ovary cells have binding activities that were almost identical with that of a pituitary derived reference h-TSH. Earlier studies for the production of monoclonal antibodies using h-TSH as antigens and fused with P3-NSI/1-Ag4-1 myeloma cells were reported by Soo and Siddle (1981), who produced 10 different antibodies from 4separate fusions. Production of monoclonal antibodies directed against different epitopes was also done by Braun and Charreire (1990), which had no cross reaction with other human glycoprotein hormones, they tried to develop Mab specific to beta subunit of the hormone using h-TSH as antigen and fused with $SP^2/0$Ag-14 myeloma cells and selected 5 different antibodies which had no cross reaction with other class of glycoprotein hormones. Other studies on the production of monoclonal antibodies specific for the alpha subunit of glycoprotein hormones have also been reported by Kofler *et al*. (1981). Minch *et al* (2004) studies on antibodies to TSH-receptor in thyroid autoimmune disease interact with monoclonal antibodies whose epitopes are broadly distributed on the receptor and they concluded that e conclude that, contrary to some reports, the binding of TSAb and TBAb to the TSH-R is not restricted to distinct and distant epitopes. The middle part of the TSH-R seems to be more relevant for TSAb binding than the N-terminal part, while a proportion of TSAb autoantibodies also binds to a C-terminal epitope of the TSH-R. In an investigation done by Mirapurkar *et al*. (2007) that some of the hTSH monoclonal antibodies

produced were polyreactive, reacting with hTSH as well as with unrelated antigen BSA, while others were monoreactive, reacting only to hTSH. However, it should be possible that many factors, including age, medicines administered and other conditions (pregnancy, some diseases), may affect TSH level.

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