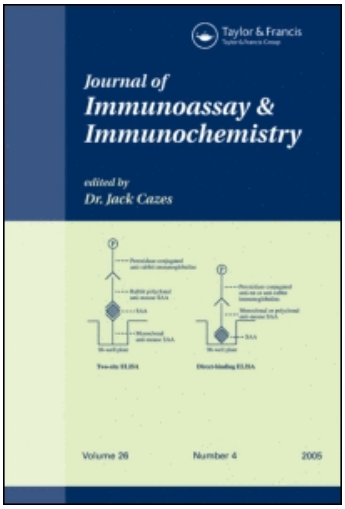

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S. Mirapurkar[a]; U. H. Nagvekar[a]; N. Sivaprasad[a]
[a] Immunoassay, Labelled Compounds & Jonaki, Board of Radiation and Isotope Technology, Department of Atomic Energy (DAE), Navi Mumbai, India

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S. Mirapurkar, U. H. Nagvekar, and N. Sivaprasad
Immunoassay, Labelled Compounds & Jonaki, Board of Radiation and Isotope Technology, Department of Atomic Energy (DAE), Navi Mumbai, India

Abstract: Monoclonal antibodies against human thyroid stimulating hormone (hTSH) are among the key reagents required in the development of an immunoradiometric assay (IRMA) for hTSH in serum. In this study we have produced and characterized twelve hTSH monoclonal antibodies. Hybridomas were generated by fusion of B-lymphocytes from mice immunized with hTSH and myeloma cells. Clones producing antibodies against hTSH were selected using ELISA (Enzyme Linked Immunosorbent Assay). Antibodies from the selected and cloned hybridoma cells were purified by affinity chromatography and their reactivities were tested by ELISA against a panel of antigens, i.e., hTSH, bovine serum albumin (BSA), and milk protein, and also by studying the binding of these monoclonal antibodies with the radioiodinated antigens (hTSH, BSA, and milk protein). It was observed 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.

Keywords: Polyreactivity, Monoreactivity, Hybridoma, hTSH monoclonal antibodies, BSA, ELISA

INTRODUCTION

Polyclonal antiserum contains a large number of antibodies directed against different epitopes on the surface of the immunogen. According to the clonal selection theory, a given lymphocyte has a unique receptor specificity and

Address correspondence to Dr. N. Sivaprasad, Board of Radiation and Isotope Technology, Sector 20, Opp. Fruit Market, Navi Mumbai 400 705, India. E-mail: shiv2011@rediffmail.com

is, therefore, committed to making only one specific antibody on appropriate stimulation. Thus, the hybridoma technique,[1] which involves the fusion between the immunogen primed spleen cells and myeloma cells, is expected to make monoclonal antibodies that are specific to one epitope of the immunogen. However, many of the hybridoma cells produced monoclonal antibodies that were not monoreactive but, instead, reacted with a variety of unrelated antigens.[2–4] A similar observation made by us during the generation of monoclonal antibodies (MAbs) for hTSH, a glycoprotein hormone secreted by the anterior pituitary, is described in this paper. Hybridomas were generated by fusion of B-lymphocytes from mice immunised with hTSH and myeloma cells. We characterized 12 monoclonal antibodies generated against hTSH, and observed that some hTSH monoclonal antibodies demonstrated the phenomenon of polyreactivity.

EXPERIMENTAL

BALB/c mice were procured from the BARC animal facility. The SP2/0 Ag-14 myeloma cell line was obtained from Radiation Medicine Centre, India. Complete Freunds adjuvant (CFA), incomplete Freunds adjuvant (IFA), hTSH, BSA, pristane, reagents for radioiodination (chloramine-T, sodium metabisulphite and potassium iodide) were obtained from Sigma, USA. ^{125}I, as sodium iodide ($Na^{125}I$), was purchased from NEN™ Life products, USA, and ELISA microtiter plates from Nunc, Rosklide Denmark. Milk protein was procured from Nestle, India and the antibody isotyping kit from Calbiochem Germany. Horseradish peroxidase labeled goat antimouse antibody, TMB/H_2O_2 (Tetramethylbenzidine/Hydrogenperoxide) substrate and goat antimouse serum were procured from Bangalore Genei, India. Protein A Sepharose was from Pharmacia fine Chemicals, USA. Polystyrene assay tubes were from Greiner, Germany.

Immunization

BALB/c mice were immunized intraperitoneally (i.p) with 10 μg of hTSH in 200 μL of phosphate buffered saline (PBS) emulsified in 200 μL of CFA. Mice were boosted i.p at 3 weeks interval with 10 μg of hTSH in 200 μL of saline solution emulsified at 1:1 with IFA. Mice showing high titers of hTSH antibody in the test bleed were given a final intravenous booster with 20 μg of hTSH dissolved in saline. Spleens of these animals were isolated 48 hr after the intravenous booster.

Monoclonal Antibodies: Generation and Purification

Spleen cells were fused to myeloma cells SP2/0-Ag-14 and cloned. Hybridoma clones producing antibodies against hTSH were identified by enzyme-linked

immunosorbent assay (ELISA) using microtiter plates coated with 1 μg/100 μL of hTSH. The presence of antibodies was identified by observation of colour after incubation with peroxidase labeled goat anti-mouse antibody and then addition of substrate. The antibodies were also confirmed by their binding to ^{125}I labeled hTSH. The selected hybridoma clones were subcloned twice by limiting dilution over a BALB/c peritoneal macrophage feeder layer. The IgG subclass of the MAbs was determined using an ELISA isotyping kit. The MAbs were produced in large amounts by injecting hybridoma cells i.p. into BALB/c mice primed with pristane. For in vitro analysis, MAbs were purified from hybridoma supernatants or ascites by protein A chromatography. Protein concentrations were determined by the method described by Lowry et al.[5] Of these, two MAbs were identified, MAb 2H11 for demonstrating polyreactivity and MAb 3B5 for demonstrating monoreactivity.

ELISA Demonstrating Polyreactivity

The identified MAbs (2H11 and 3B5) were tested for their reactivity with the antigens, hTSH, BSA (bovine serum albumin), and milk protein. Microtiter plates were coated with 10 μg/mL of each antigen in 100 μL of 0.1 M sodium bicarbonate buffer, pH 9.6, overnight at 4°C. After washing the plates with phosphate buffered saline-Tween (PBS-T), [0.01 M phosphate buffer. pH 7.2, containing 0.15 M NaCl and 0.1% Tween-20], unbound sites on the tube surface were blocked by incubating, in the tube, 1% milk protein in PBS-T at 37°C for 1 hr. Increasing concentrations of the MAbs (2H11 and 3B5) in PBS-T were added to the wells and incubated at 37°C for 2 hr. After five washings with PBS-T, the plates were incubated at 37°C for 2 hr with peroxidase labeled goat anti-mouse antibody (1:500) in PBS-T. The plates were washed and the colour developed with TMB/H_2O_2 substrate. After 10 min, the reaction was quenched with 2M H_2SO_4 and the absorbance values were read at 450 nm.

Competitive Inhibition ELISA and Determination of Affinity Constant

The following competitive inhibition ELISA procedure was used to further demonstrate the reactivity of the identified MAbs to the antigens. 200 μL of the MAbs (2H11 and 3B5), at a concentration of 100 μg/mL, were pre-incubated with 200 μL of various concentrations of the antigens (0.1, 1, 10, and 100 μg/mL). After 24 hr incubation at 25°C, 150 μL of each mixture was transferred and incubated for 1 hr at 25°C into the wells of a microtitre plate previously coated with 150 μL of hTSH (10 μg/mL). This ELISA procedure was also used for determining the affinity constant (K) of the MAbs; the K values were calculated according to the method of Friguet.[6]

Antibody Specificity Studies using Radioiodinated Antigens and MAbs

Polyreactivity was also demonstrated using radioiodinated MAbs and antigens. 500 μL of various concentrations (0.1, 1, 10, and 100 μg / mL) of the antigens hTSH, BSA, and milk protein in 0.1 M sodium bicarbonate buffer, pH 9.6, were immobilized on the polystyrene tubes by incubating for 24 hr at 25°C. After extensive washings with PBS-T, unbound sites were blocked with 1% milk protein solution in PBS-T. To the washed tubes, 500 μL of the radioiodinated MAbs (80,000 cpm/tube) was added and incubated for 24 hr at 25°C. The tubes were then washed and the associated radioactivity was measured in the NaI (Tl) scintillation counter.

100 μL of the radioiodinated (40,000 cpm/tube) antigens (hTSH and BSA) were incubated with 100 μL of various concentrations (0.01, 0.1, 0.5, and 1 μg/mL) of the MAbs with 10 μL of normal mouse serum for 24 hr at 25°C. The bound fraction was precipitated by adding 100 μL (1:1,000) of goat anti-mouse serum and 1 mL of polyethylene glycol (PEG) 6000 (10%). The mixture was centrifuged at 3,000 rpm for 20 min, and the radioactivity associated with the pellet was measured in the NaI(Tl) scintillation counter.

RESULTS AND DISCUSSION

In this study, we isolated 12 hTSH reactive MAbs. The isotypes of these MAbs are shown in Table 1, along with their reactivities towards hTSH and BSA. It can be seen that the MAbs of different isotypes showed polyreactivity without any particular isotype predominating. Four MAbs showed reactivity to both

Table 1. Reactivity of purified MAbs with hTSH and BSA using anti-mouse–HRP conjugate

Antibody	Isotype	hTSH[a]	BSA[a]
2C11	IgG2a	+	−
2E3	IgM	+	−
2H11	IgG1	+	+
2B11	IgG1	+	−
3B5	IgG2a	+	−
6G7	IgG2b	+	−
6G3	IgG1	+	−
6G10	IgM	+	−
5D4	IgG1	+	−
1D10	IgG2a	+	+
4B5	IgG3	+	+
6A5	IgM	+	+

[a]hTSH/BSA were coated on the microwells.

hTSH and BSA, while others reacted only to hTSH. MAb 2H11 was taken for further evaluation of the polyreactivity of the MAb. For comparative studies, the MAb 3B5, not showing polyreactivity, was taken. The ELISA (Fig. 1a) shows a binding of MAb 3B5 with only hTSH, while MAb 2H11 shows binding with both hTSH and BSA. For confirmation of antibody specificity, inhibition experiments using competitive enzyme immunoassay was performed. The MAbs at 100 μg/mL concentrations were preincubated for 2 hr at 37°C with increasing concentrations of the three antigens. The results are shown in Fig. 1b; binding of MAb 3B5 is inhibited only by hTSH, while the binding of MAb 2H11 is inhibited by hTSH and BSA. As shown in Table 2, the affinity constant of hTSH for MAb 2H11 (1.6×10^7 L/Mol) is lower than that of MAb 3B5 (1.2×10^{10} L/Mol), which is in agreement to the published reports.[3,4,7] which state that the affinity of a

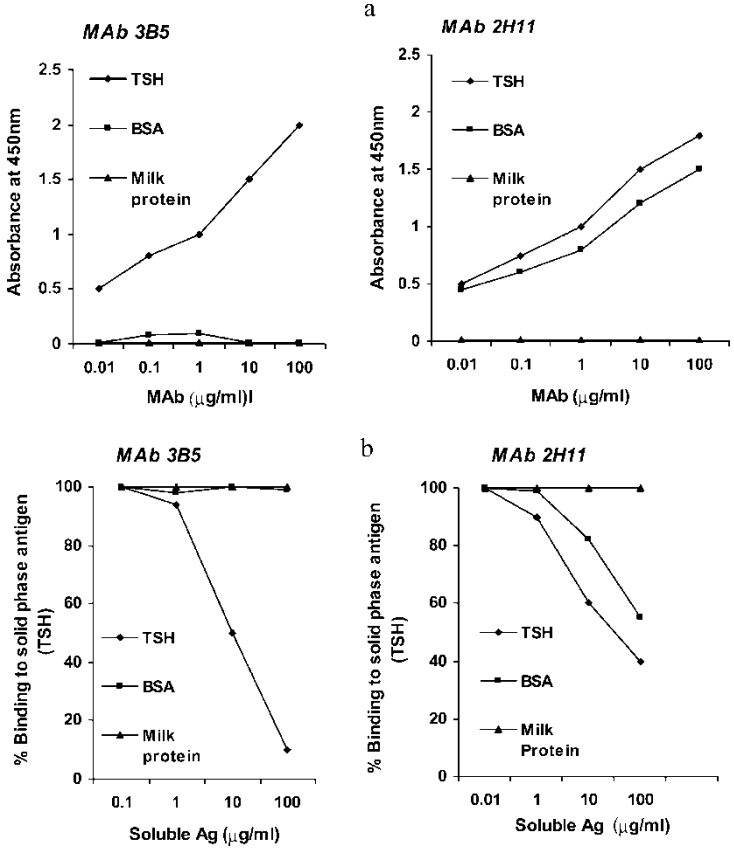

Figure 1. a) ELISA binding of the polyreactive MAbs (2H11) and monoreactive (3B5) to different antigens. b) Competitive inhibition study using ELISA for both the MAbs (2H11 and 3B5) with the antigens.

Table 2. Affinity constant values (K) in L/Mol of hTSH MAbs for hTSH and BSA

Antibody	hTSH	BSA
Monoreactive 3B5	1.2×10^{10}	—
Polyreactive 2H11	1.6×10^{7}	1.2×10^{7}

polyreactive antibody for different antigens was, in general, lower than that of a monoreactive antibody. The reactivity studies with radioiodinated MAbs is shown in Fig. 2a. The binding of radioiodinated MAb 2H11 increased when hTSH and BSA concentrations used for coating were increased from

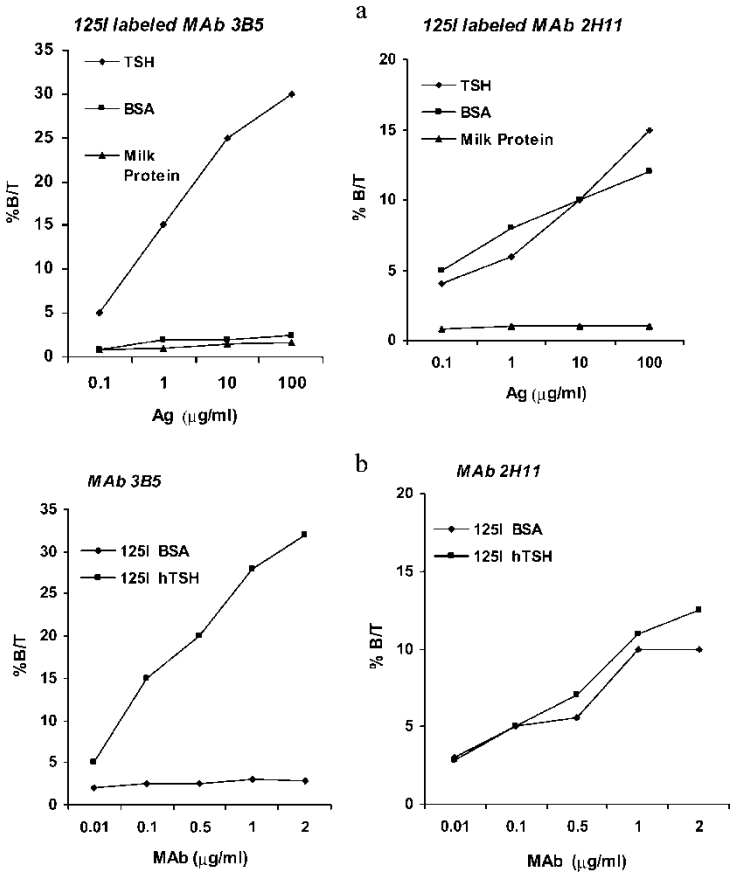

Figure 2. a) Reactivity studies of the radioiodinated polyreactive MAbs (2H11) and monoreactive (3B5) with varying concentrations of antigens (hTSH, BSA, and milk protein) immobilized on to the polystyrene tubes. b) Binding of the radioiodinated antigens (hTSH and BSA) with the increasing concentrations of MAbs (2H11 and 3B5).

0.1–100 μg/mL, while no increase was registered for milk protein. Figure 2b shows the reactivity of the MAbs with the radioiodinated antigens. An increase in the binding of both radiolabeled hTSH and radiolabeled BSA was observed with increasing concentrations of the MAb2H11. In the case of MAb 3B5, an increase in the binding was observed only with radiolabeled hTSH, while there was no binding observed with radiolabeled BSA. All of these studies confirm the polyreactivity of MAb 2H11.

The phenomenon of polyreactivity of an antibody molecule can be explained by a possible conformational change taking place in the antibody molecule at close proximity to the antigen.[4] Polyreactive antibodies possess many of the characteristics ascribed to human and mouse 'natural' antibodies and can be of IgM, IgG, and IgA isotypes.[2] In this study the polyreactive antibody had K value (Table 2) of 10^7 L/Mol to hTSH, while the monoreactive antibody had K value of 10^{10} L/Mol towards hTSH, thus showing that the monoreactive antibody had a higher affinity for the immunizing antigen (hTSH). In order for the polyreactive antibody to bind to two structurally unrelated antigens, in this case hTSH and BSA, the binding site of the antibody needs to be more flexible. This may lead to poor binding of the antigen and, thus, low affinity for the antigen. This is reflected in the low affinity constant of the polyreactive antibody (MAb 2H11) for hTSH. For the development of an immunoassay, it is important to identify and eliminate, at earlier stages, the polyreactive antibodies to avoid artifacts in the assay due to their reactivity to unrelated antigens. Hence, the clones need to be screened for their reactivity to unrelated antigens which are likely to be encountered in the assay and samples.

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REFERENCES

1. Kohler, G.; Milstein, C. Continuous culture of fused cells secreting antibody of predefined specificity. Nature **1975**, *256*, 495–497.
2. Barrach, H.J.; Chichester, C.; Chichester, A.; Matoney, A.; Srinivas, G.R. Evidence for polyreactivity seen with monoclonal antibodies produced against type II collagen. J. Immunol. Meth. **1991**, *140* (24), 259–267.
3. Casali, P.; Notkins, A.L. Probing the human B-cell repertoire with EBV: polyreactive antibodies and CD5+ B lymphocytes. Ann. Rev. Immunol. **1989**, *7*, 513–535.

4. Notkins, A.L. Polyreactivity of antibody molecules. Trend. Immunol. **2004**, *25* (4), 174–178.
5. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **1951**, *193*, 265–274.
6. Friguet, B.; Chaffotte, A.F.; Djavadi-Ohaniance, L.; Goldberg, M.E. Measurement of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunoadsorbent assay. J. Immunol. Meth. **1985**, *77*, 305–319.
7. Ternyck, T.; Avremeas, S. Murine natural monoclonal autoantibodies: a study of their polyspecificities and their affinities. Immunol. Rev. **1986**, *94*, 99–112.

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