Original Article

Production and Characterisation of Monoclonal Antibodies against 19-Nortestosterone*

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Abstract

Objective To produce anti-19-Nortestosterone (NT) monoclonal antibodies and identify their immunological characteristics.

Methods Hybridomas were prepared by fusing NS0 mouse myeloma cells with splenocytes isolated from immunized BALB/c mice. Noncompetitive and competitive indirect ELISA were employed to screen positive cell clones. A caprylic acid ammonium sulphate (CAAP) method was used to purify NT mAb, and the Batty saturation method was used to determine the affinity constant (Kaff).

Results Five hybridoma cell lines, named NT-1, NT-2, NT-3, NT-4, and NT-5, were identified and their corresponding mAbs were of the IgG_1 isotype with a *k* light chain. The Kaffs of all mAbs were between 2.6 and 4.7×10^9 L/mol. The titers and IC_{50} values of purified ascite fluids were in the range of $(0.64–2.56)\times10^5$ and (0.55–1.0) ng/mL, respectively. Of all the cross-reacting steroids, α-NT was the most reactive with the mAbs at 62% with NT-1 mAb and 64% with NT-2 mAb. Negligible cross-reactivity (<0.01%) with other steroids was observed.

Conclusion The establishment of these hybridomas allows the potential development of a rapid test kit, and may provide an alternative method for the detection of NT residues in food producing animals.

Key words: 19-Nortestosterone; Hybridoma cell lines; Monoclonal antibody; Characterization; Identification

Biomed Environ Sci, 2011; 24(2):172-179 doi:10.3967/0895-3988.2011.02.012 ISSN:0895-3988

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INTRODUCTION

Nandrolone (19-Nortestosterone, NT; 17β-hydroxyestra-4-en-3-one) is an endogenous chemical discovered in the 1930s that is found in a number of species[1]. It is a xenobiotic with androgenic and anabolic properties. In the 1950s, recognition of the growth promoting property of NT led to its introduction as a tool to accelerate meat production that can enhance lean tissue growth, reduce fat deposition, increase weight gain and feed conversion efficiency of animals[2-4]. Administration is usually by intramuscular injection of the esterified drug in suspension with benzyl alcohol and *Arachis* oil, or inserted into the fleshy part of the ear. The hormones are gradually released over a period of 50 days or so, to ensure a relatively constant and slightly elevated level in the blood[5]. NT has also been employed as a doping agent to increase muscle mass and physical strength that provides a boost in the physical performance of athletes in sports and horseracing because of its androgenic activity[4,6].

Despite these apparent benefits, studies show

*This work was supported by the Henan Innovation Project for University Prominent Research Talents, 2010HASTIT026.

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Received: February 21, 2010; Accepted: January 12, 2011

that NT and its metabolites in meat produce a series of adverse effects, including peliosis hepatitis, hypoproteinemia, adrenal atrophy, dyssecretosis, decreased semen production, testicular shrinkage, menstrual cycle disorders, masculinization, cerebral dysfunction, emotional instability, and other side effects[7-10]. Thus, the use of natural and synthetic hormones for growth promotion purposes in meat-producing animals has been prohibited in the European Community since 1986, in order to protect consumers from possible developmental, neurobiological, genotoxic, and carcinogenic effects[11]. NT and its esters, as well as metabolites are also prohibited in food animal veterinary drugs and other banned compounds lists in China (Notice No. 193 of the Ministry of Agriculture in April 2002). However, illegal use of NT as a growth promoter has been widely reported throughout Europe[2] and in China, thus prompting continuous surveillance to control its abuse.

Traditionally, NT residue analysis has relied upon classical analytical methods, such as gas chromatography coupled with mass spectrometric (GC–MS)[6, 12-14], liquid chromatography coupled with mass spectrometric (LC–MS)[15-17], and other quantitative methods. Chromatographic techniques generally require highly skilled personnel, laborious sample pretreatment, complex equipment and high-cost, and are therefore not suitable for routine analysis of a large number of samples or on-site determinations. Immunological techniques are increasingly considered as alternative and/or complementary methods for residue analysis, because of their simplicity, cost-effectiveness, portability, and high sample throughput.

Therefore, our main goal was to produce high-sensitivity monoclonal antibodies (mAbs) displaying excellent affinity and specificity towards NT, and to optimize ELISA protocols based on selected monoclonal antibodies. This work potentially optimizes the pre-treatment procedures for LC–MS and GC–MS detection, and provides reagents for the development of NT kits and test strips.

MATERIALS AND METHODS

Materials and Chemicals

Estradiol, 19-Nortestosterone (NT), 17a, 19-Nor testosterone (a-NT), Trenbolone, Methyltestoster one, and Clostebol were purchased from Dr Company (Germany). NT-17- (succinic anhydride) ester-BSA (NT-17-BSA) was used as an immunogen and NT-3-carboxymethyloxime-BSA (NT-3-BSA) was used as a coating antigen, and both were conjugated in our laboratory. Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) were obtained from Pierce Biotechnology, Inc. (Rockford, II, USA). GaMIgG-HRP (whole molecule specific) was purchased from Sino-American Biotechnology Company (Shanghai, China). Transparent 96-well polystyrene microtitre plates (Boyang Experimental Equipment Factory, Jiangsu, China) were used for colorimetric measurement. Hypoxanthine/thymid ine/aminopterin (HAT) and hypoxanthine/thymidine (HT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 with L-glutamine was obtained from Invitrogen. Polyethylene glycol 1500 (PEG 1500, 50%) was from Roche Diagnostics Corporation (Indianapolis, IN, USA). Foetal bovine serum (FBS) was from Hangzhou Sijiqing Biological Engineering Materials Co. Ltd. (Hangzhou, China). Cell culture plates (24- and 96-wells) and culture flasks were obtained from Costar Inc. (Bethesda, MD, USA). A mouse monoclonal antibody isotyping kit was purchased from Pierce Biotechnology, Inc. (Rockford, II, USA). Phenacetin, 3,3,5,5-tetram ethylbenzidine (TMB), and urea peroxide were obtained from Sigma Co. (St. Louis, MO, USA). All other solvents and reagents were of analytical grade or higher, unless otherwise stated.

Eight-week-old female BALB/c mice were obtained from the Laboratory Animal Center (Beijing Medical University, China) and kept under strictly controlled conditions in our laboratory facility.

Buffers

The buffers used were as follows: phosphate buffered saline (PBS) consisting of NaCl (137 mmol/L), $Na_2HPO_4.12H_2O$ (10 mmol/L), KCl (2.68 mmol/L), and KH_2PO_4 (1.47 mmol/L), pH 7.4; Carbonate buffered saline (CBS) contained Na_2CO_3 (15 mmol/L), $NaHCO_3$ (35 mmol/L), pH 9.6; Washing buffer consisted of PBS containing 0.05% Tween-20; Blocking buffer contained BSA (1% w/v) in PBS. Substrate buffer consisted of mixing part A (500 mL) and part B (500 mL) solutions. Part A contained (per 1 L of water) citric acid (15 mmol/L), anhydrous sodium acetate (85 mmol/L), Phenacetin (0.45 mmol/L) and urea peroxide (0.5 mmol/L), adjusted to pH 5.0 with HCl. Part B contained TMB (4 mmol/L) dissolved in 500 mL of methanol and 500 mL of glycerol. The stopping solution was 2 mol/L H_2SO_4.

Instruments

A spectrophotometric microtitre plate reader (MULTISKAN MK3, Thermo Scientific, USA), provided with a 450 nm filter, was used for absorbance measurements. Incubations were performed in a DH-360A oven from Zhongxingweiye Instrument Co., Ltd (Beijing, China).

General ELISA Procedures

Antibody titers were tested by indirect ELISA using the procedure described below. Microtitre plates were coated overnight at 4 °C with NT-3-OVA coating antigen at 2 μg/mL (100 μL/well). Plates were washed three times with PBST and blocked with 250 μL/well blocking buffer, followed by incubation for 2 h at room temperature. The solution was then discarded, and plates were washed three times with washing solution. Antisera were added at 50 μL/well of the appropriate dilution, and the plates were incubated for 15 min at 37 °C. After another washing procedure, GaMIgG-HRP (1:1000, 50 μL/well) was added, followed by incubation for 25 min at 37 °C. The plates were washed, and 60 μL/well freshly prepared substrate solution was added. After incubating at room temperature for 15 min, the reaction was stopped using 2 mol/L H_2SO_4. The absorbance in each well was measured at 450 nm and the antibody titre was defined as the reciprocal of the dilution that resulted in an absorbance value that was twice that of the background. Absorbance values were corrected by blank well readings (wells in which no primary antibody was added).

Indirect competitive ELISA (icELISA) was employed to determine the sensitivity and specificity of the assay. Checkerboard tests were performed to determine the optimal dilution of the coating antigen and the primary antibody, resulting in the following optimized protocol. Ninety-six well polystyrene microtitre plates were coated with 100 μL per well of OVA-conjugate solution, then received 50 μL per well of analyte in PBS containing 5% methanol plus 50 μL per well of hybridoma supernatant or purified antibody solution in PBST, unless otherwise stated. All samples were run in triplicate wells. The following procedure was followed as described for the indirect ELISA. With the inhibition ELISA format, analytes that do not react with the antibody would produce absorbance near 100%; conversely, analytes that do react with the antibody would decrease in percentage of absorbance. The inhibition rate was expressed as $\%B/B0$, where B is the absorbance of the well containing competitor and $B0$ is the absorbance of the well without competitor. Standard curves were calculated by mathematically fitting experimental points to a four-parameter logistic equation.

Immunization Schedule

Five BALB/c female mice (8-10 weeks old) were immunized with NT-17-BSA conjugates by subcutaneous injections at multiple sites. The first dose consisted of 60 μg of immunogen as an emulsion of PBS and Freund’s complete adjuvant. Three subsequent injections were given at three-week intervals with the same dosage of immunogen emulsified in Freund’s incomplete adjuvant. After a resting period of at least three weeks from the last injection with adjuvant, mice were tail-bled and screened for anti-NT activity by icELISA. The mouse showing the highest anti-NT activity received a final soluble intraperitoneal (ip) injection of 100 μg of conjugate in PBS, 3−4 days prior to the cell fusion procedure.

Fusion of Myeloma and Spleen Cells

Portions of the cell fusion procedures and cloning conditions were previously described by Köhler and Milstein[18] with further modifications by Chen et al.[19]. Briefly, NS0 myeloma cells were passed through medium containing 8-azaguanine and then grown for 4−5 days at 37 °C in a 5% CO_2 atmosphere. On the day of fusion, the mouse that received the booster injection was sacrificed by cervical dislocation and the spleen was removed aseptically. Splenocytes were isolated and fused with myeloma cells at a 10:1 ratio using PEG 1500 as the fusing agent, followed by gentle stirring for 1 min. The resulting mixture was kept still at 37 °C for 90 s, and then 40 mL of HAT-1640 medium (supplemented with 15% FBS) was slowly added to the fused cells. The fused cells were then distributed into 96-well culture plates, in which mouse peritoneal macrophages were prepared on the day before the fusion and were grown with the selective HAT medium.

Hybridoma Production, Selection, and Cloning

At 10-14 days after fusion, supernatants of hybridomas were recovered and screened using a combination of noncompetitive and competitive indirect ELISA. Well cultures showing significant NT recognition activity were expanded from the culture

in the 96-well plate to a 24-well plate, and subcloned three times by limiting dilution. The wells picked for expansion were viewed under a microscope to confirm the presence of a single cell source, ensuring their monoclonal origin, and the HT medium was gradually replaced by complete medium. After hybridomas became dense in the 24-well plate, they were transferred to 50 or 100 mL culture flasks. Hybridoma cells were collected, centrifuged, and the supernatants were stored at −20 °C until used. Colonies of interest were frozen in culture medium containing 10% dimethyl sulphoxide (DMSO) and cryopreserved in liquid nitrogen, followed by defrosting three times to select the stable antibody-producing clones.

Monoclonal Antibody Production

A mature female BALB/c mouse was injected intraperitoneally (i.p.) with 0.5 mL paraffin 10 days before receiving an i.p. injection of the positive hybridoma cells suspended in RPMI 1640 medium. Ascites fluid was collected 10 days after the injection and then stored at –20 °C. Purification of mAb was performed according to the modified caprylic acid, ammonium sulphate precipitation (CAASP) method described previously[20].

Characterization of mAbs

The purified antibody was immediately used for the characterization. The protein content of the antibody was determined according to the following formula: protein concentration (mg/mL)=1.45OD_{280}-1.74OD_{260}, where OD value is the optical density[21]. Measurement of monoclonal antibody affinity (*K*a) was performed according to the procedure described by Wang et al.[22]. The class and subclass of the isotypes of the purified antibody were determined using a mouse monoclonal antibody isotyping kit. The calibration curves were fitted based on the average of three independent assays in triplicate and the IC_{50} values were calculated to determine the sensitivity, which represented the concentration of NT that produced 50% inhibition of antibody binding to the hapten conjugate. Specificity was defined as the ability of structurally related chemicals to bind to the specific antibody[23] and cross-reactivity (CR) were calculated as: [(IC_{50} of NT)/(IC_{50} of chemicals)]×100. Therefore, the lower the CR, the higher the specificity of NT mAb.

Application of mAbs

Fresh samples of swine meat that had not been exposed to steroids were purchased from retail outlets in Xinxiang, China. The samples were homogenised with a high-speed triturator, and collected in a 50 mL round-bottomed plastic flask. Precisely 2 g of the sample homogenate was accurately weighed into a glass centrifuge tube and NT standard solution was added at this step to produce spiked levels of 2, 4, 6, 8, 10, and 12 ng/g tissue. The spiked sample was mixed thoroughly, allowed to stand at room temperature for 2 h, and then a volume of 5 mL acetate buffer (0.2 mol/L; pH 6.5) was added. The mixture was subjected to enzymatic hydrolysis with 50 µL of β-glucoronidase from *E. coli* (Sigma-Aldrich, USA) and incubated on an oscillator at 37 °C for 6 h. After 5 mL of methanol was added, the sample was mixed briefly and centrifuged at 5 000 rpm for 10 min. The supernatant was diluted with 0.01 mol/L PBS, and then applied to the mAb-based icELISA for analysis. A correlation was identified between concentration spiked and concentration determined by icELISA.

RESULTS

Selection for Potential Cell-fused Mice

Five mice were immunized with the NT-17-BSA conjugate following the standard protocols described above, while NT-3-OVA were coated onto ELISA plates to determine the titre and inhibition level of antisera. After three subsequent injections, four of the five mice immunized with NT-17-BSA produced antisera with significant anti-NT activities (Figure 1). From the obtained inhibition curves, we determined that mouse No. 4 afforded the most sensitive IC_{50} value (17.5 ng/mL) and selected it for further study.

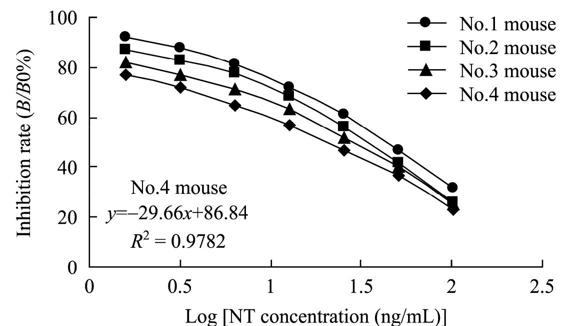

Figure 1. PCR Inhibition curves of NT antisera against NT by indirect competitive ELISA.

Production of Monoclonal Antibodies

10 days following the cell fusion procedure, growing hybridoma cell clones could be observed in many wells of the seeded 96-well plates. The fusion rate of the mouse spleen cells with myeloma cells was approximately 85%. Supernatants of all wells were screened by simultaneous non-competitive and competitive assays, and the positive well rate was 18%. Selection of clones from these positive cultures by limiting dilution led to five stable hybridoma cell lines. These monoclonal cultures and their corresponding cell lines were named NT-1, NT-2, NT-3, NT-4, and NT-5. Using a mouse monoclonal antibody isotyping kit, all five antibodies were determined to be of the IgG_1 isotype with a *k* light chain; the protein concentrations of all mAbs were between 5.6–8.4 mg/mL. Finally, the five hybridomas were expanded and stored in liquid nitrogen. The mAbs from culture supernatants and ascite liquids were purified and characterized. Based on the results of the checkerboard titration, the antibody titres and IC_{50} values were determined (Table 1). From the obtained inhibition curves, the hybridomas that produced the most sensitive antibodies (NT-1 and NT-2) produced IC_{50} values of 0.55 and 0.58 ng/mL, respectively. These data suggest that the sensitivity of mAb increased approximately 30-fold in comparison to that of the NT polyclonal antibody (pAb) tested previously. These two hybridomas were selected for further evaluation of mAb specificity and subsequent immunoassay development.

Table 1. The Titre and IC_{50} of mAbs Produced by Five Hybridomas

Monoclonal Antibody	Titre of Culture Supernatants	Titre of Ascite Liquids [a]	IC_{50} Values of Ascite Liquids (ng/mL) [b]
NT-1	5.12×10^2	2.56×10^5	0.55
NT-2	5.12×10^2	2.56×10^5	0.58
NT-3	2.56×10^2	1.28×10^5	0.8
NT-4	1.28×10^2	1.28×10^5	0.93
NT-5	1.28×10^2	0.64×10^5	1.0

Note. [a]Indirect ELISA curves are shown in Figure 2. [b]Standard curves for the sensitive values are illustrated in Figure 3.

Hybridoma Stability Experiments

Five hybridoma cell lines producing antibodies were identified by stability verification. Cultures were subjected to a freeze/thaw procedure three times and the performance of NT-1 and NT-2 hybridomas were more consistent in the experiments (Figure 4).

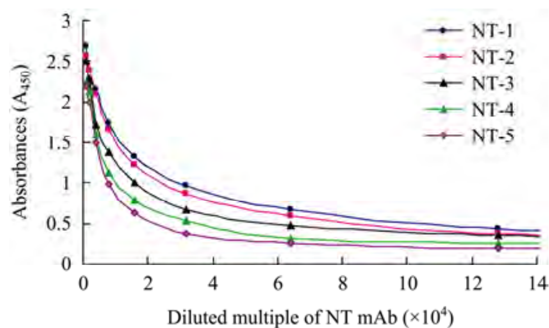

Figure 2. NT mAb indirect ELISA curves. Values shown are the mean of three independent assays (*n*=3).

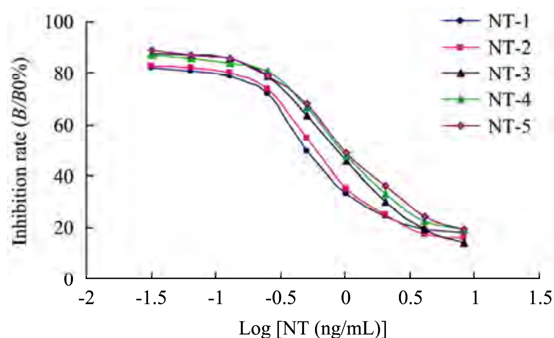

Figure 3. Inhibition curves of NT mAb by icELISA. The NT was prepared in PBS containing 5% methanol.

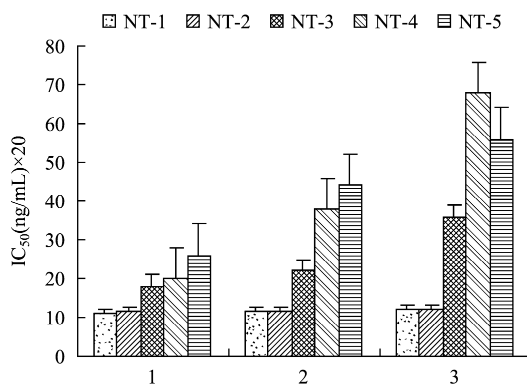

Figure 4. IC_{50} values of NT mAb generated from five hybridomas after three freeze/thaw cycles. Each data was calculated from triplicate assays.

Determination of the NT mAb Affinity Constant (Kaff)

The affinity constants for each of the five selected hybridomas were measured by ELISA using serial dilutions of both the coated antigen and NT mAb. The optimal condition of the ELISA in which the OD value was approximately 1.0 was selected. The apparent Kaffs of NT-1, NT-2, NT-3, NT-4, and NT-5

mAb were 4.7×10^9, 4.2×10^9, 3.9×10^9, 2.9×10^9, and 2.6×10^9 L/mol, respectively (Figure 5).

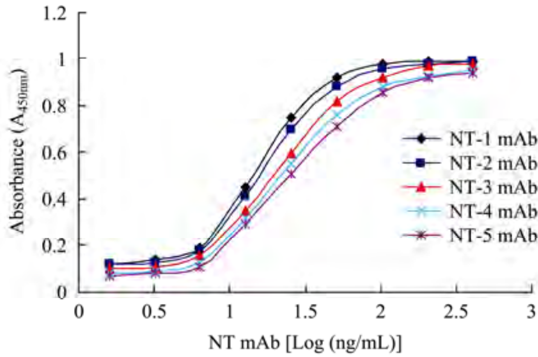

Figure 5. Affinity constant (Kaff) curves of anti-NT mAbs.

Cross-reactivity of NT Analogs

Investigations on the cross-reactivity of the obtained antibodies are crucial for the assessment of results. Therefore, a number of compounds structurally related to NT were tested by icELISA to characterize the properties of the antibody (Table 2). Of all the cross-reacting steroids, α-NT had the highest cross-reactivity, 62% with NT-1 and 64% with NT-2. Negligible cross-reactivity (<0.01%) with othersteroids was observed.

Validation of the mAb-based ELISAs

To further demonstrate the capability of mAbs for the determination of NT residues in animal tissues, two icELISA standard curves were developed using the NT-1 and NT-2 mAbs. The accuracy of the analysis was studied by the comparative detection of fortified NT in swine muscle samples at different concentrations, and the measurement correlations between the fortified and analysed concentrations determined. Figure 6 shows that data points were distributed on both sides of the trend line, where the regression equation for NT-1 based ELISA was y=0.9343x+0.1933, with a correlation coefficient (R^2) of 0.9975, and the regression equation for NT-2 based ELISA was y=1.0086x+0.04, with R^2 value of 0.9959. These data indicate that an excellent correlation exists between spiked concentration and determined concentration, and the results also demonstrate the utility of the icELISA method for detecting NT residues in animal tissues.

Table 2. Cross-reactivity of Structurally Related Steroids in the NT Immunoassay

Compounds	Structures	NT-1 mAb		NT-2 mAb	
		IC_{50} (ng/mL)	CR (%)	IC_{50} (ng/mL)	CR (%)
Nortestosterone		0.55	100	0.58	100
α-Nortestosterone		0.88	62	0.91	64
Estradiol		>5500	<0.01	>5800	<0.01
Trenbolone		>5500	<0.01	>5800	<0.01
Methyltestosterone		>5500	<0.01	>5800	<0.01
Clostebol		>5500	<0.01	>5800	<0.01

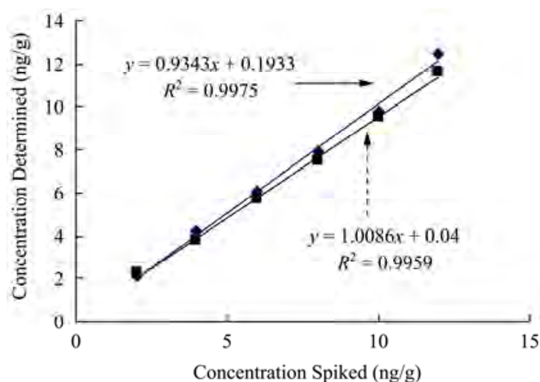

Figure 6. Correlation between spiked concentration and determined concentrations of NT in swine muscle samples fortified with NT. (◆)NT-1 mAb-based icELISA; (■)NT-2 mAb-based icELISA.

DISCUSSION

Binding capacity, sensitivity, affinity, and specificity determine the main characteristics of antibodies, and immunoassays are based on the reciprocal conjugating activity between antigen and antibody. A polyclonal antibody (pAb) usually contains unrelated or other antigenic immunoglobulin, as well as a mixture of products induced by different antigenic determinants. Therefore, the variance in the quality of ELISA kits is a common problem of the immunoassay method. The need for a homogeneous and unlimited supply of antibodies can be met using hybridoma technology that was first established by Köhler and Milstein in 1975[18]. Using this technology, five hybridoma cell lines were identified in the current study, in which two constant strains, named NT-1 and NT-2, had the highest titre (1: 2.56×10^5) and sensitivity (0.55 and 0.58 ng/mL).

The affinity constant (Kaff) of an antibody plays an important role in the quality of NT mAb. The Kaff demonstrates the conjugating ability between antibody (Ab) and hapten, or one antigen determinant, and is a significant indicator of antibody stability. Common methods used to measure Kaff, include equilibrium dialysis, Scatchard plot, Batty saturation method, competitive binding, precipitation method. The Batty saturation method employed in this article is simple, rapid, and reliable. The use of serial dilutions of mAb resulted in a sigmoid curve of OD versus logarithm of total mAb added to the well. Comparison of the OD-50 for different antibodies was a reflection of the relative number of epitopes on the Ag that were identified by different antibody paratopes, provided excessive mAb was used. Using the five identified anti-NT mAbs to measure Kaff, satisfactory results were obtained. According to James[24], the five hybridomas all produced high-affinity antibodies (between 10^7 and 10^{12} L/mol).

For the development of an immunoassay to detect NT and NT residues, it is essential to produce highly molecular or structurally specific antibodies to the NT hapten. In the current study, heterologous icELISA was employed to judge the cross-reactivity of two NT-1 and NT-2 mAbs, in which the hapten-OVA conjugate used for plate coating was synthesized by a mixed-anhydride technique, and the immunogen was prepared by employing a C-17 coupled steroid-protein conjugate through the EDC method, thus overcoming unwanted cross-reactivity (nonspecific binding)[25-26]. It is believed that the hapten- or antigen-antibody interaction is dependent on spatial structure defined by molecular shape and geometric complementary binding, and on low-energy interactions, such as hydrogen bonding, hydrophobic interaction, electrostatic or dipole–dipole forces. The data obtained in the current study for cross-reactivity were not surprising, considering that α-NT and NT have very similar chemical structure, while other analogues have an extra alkyl group, double bond or chlorine atom on the cyclopentanoperhydro-phenanthrene, resulting in obvious steric hindrance.

In conclusion, here we produced five hybridomas that produced mAbs of varying reactivity. Two mAbs were selected for the development of a highly sensitive and specific immunoassay that was able to detect NT and α-NT in spiked swine meat homogenates. This study shows that the development of these reagents is likely to lead to the production of highly sensitive assays to monitor NT use not only in the food industry, but perhaps in dope testing of racehorses or humans as well.

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