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Monoclonal Antibodies Against Male-Specific Antigen of White Lamphun Cattle (*Bos indicus*)

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ABSTRACT

A male-specific antibody was used in a cytotoxic reaction to increase X sperm in whole sperm by lysing Y sperm. The objective of this study was to produce a monoclonal antibody against male-specific antigen of White Lamphun cattle. From this study, monoclonal antibodies were produced by biweekly immunization of 3 Balb/c mice with 10×10^6 cells of White Lamphun cattle sperm. Mouse number 2 was chosen to be used in the fusion process by fusing myeloma cells and splenocytes from the immunized Balb/c female mouse with a ratio of 3:1 (splenocyte:myeloma) and the result from the enzyme-linked immunosorbent assay (ELISA) showed that hybridoma cells named P1C2 produced antibody amounts against male and female white blood cells of White Lamphun cattle of 0.55 and 0.38 optical density (O.D.) units. P1C2 was used in the cell limiting process, evaluated using antibody titer and resulted in positive clones named P1C2B8 and P1C2C9. The ELISA results showed that P1C2B8, P1C2C9 and P1C2B8&C9 produced antibody amounts against male and female white blood cells of 1.74 ± 0.03 and 1.56 ± 0.05, 1.04 ± 0.03 and 0.88 ± 0.03 and 1.47 ± 0.07 and 1.27 ± 0.07 O.D. units, respectively. The results from immunofluorescence showed that P1C2B8, P1C2C9 and P1C2B8&C9 produced antibody amounts against male and female white blood cells of 92.15 ± 0.87 and 69.33 ± 0.93, 91.19 ± 0.95 and 72.95 ± 1.27 and 93.27 ± 1.25 and 68.68 ± 1.23 %, respectively ($P<0.05$). It was concluded that the monoclonal antibodies produced in this study were specific to the male more than the female.

Keywords: specificity, monoclonal antibodies, white blood cells, White Lamphun cattle, *Bos indicus*

INTRODUCTION

In the upper north of Thailand, there is a native cattle breed called White Lamphun (*Bos indicus*). The cattle of this breed are well adapted to tropical environments and are highly fertile with low nutritional requirements. However, changes in the agricultural system have resulted in decreased numbers of these cattle. Sex selection before or during conception is one of the techniques used to increase the number of cattle. There are several methods used to sex cattle to achieve the desired gender of offspring. An example of an invasive method that affects the embryos is embryo biopsy for sexing by polymerase chain reaction (PCR) (Lopes *et al*., 2001; Park *et al*., 2001) while an example of a non-invasive method that does not affect the embryos is the application of male-specific histocompatibility-Y antigen (H-Y antigen) which is specific to the male embryo membrane and stops the development of male embryos (Ramalho *et al*., 2004). Nowadays, sexing before fertilization has been developed because it reduces the amount of time required

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and also reduces damage to embryos in the biopsy process that causes a low pregnancy rate in cows. One such technique involves separating X-chromosome-bearing sperm (X sperm) and Y-chromosome-bearing sperm (Y sperm) by flow cytometry which is widely used in artificial insemination to increase the numbers of the desired sex of calves. Flow cytometry is a tool to separate sperm according to their DNA content with great accuracy (Seidel Jr, 2007). X sperm is larger than Y sperm because X sperm has a greater DNA content than Y sperm which leads X sperm to absorb a greater amount of fluorescent dye than Y sperm and when evaluated under UV light, X sperm fluoresces brighter than Y sperm, so X sperm and Y sperm are separated during the flow cytometry process (Hendriksen *et al.*, 1993). However, flow cytometry has some disadvantages, such as a lower fertilization rate because of the sperm damaged through the process (Guthrie *et al*., 2002; Seidel Jr., 2007). Furthermore, the success of using sexed sperm requires expensive equipment, high-skilled technicians and suitable inseminators that make the application of H-Y antigen a challenging option. The application of H-Y antigen has been used in the production of an antibody against H-Y antigen and is called H-Y antibody and its use via cytotoxic reactions results in H-Y antibody combining with a complement protein to lyse Y sperm (Goldberg *et al*., 1971; Bennett and Boyse, 1973; Shelton and Goldberg, 1984). While the sexing efficiency of antibodies is less than that of flow cytometry, the advantages of the former-mentioned technique are the low cost and the greater speed of sexed semen production. Studies of the male-specific antigen (H-Y antigen) have been widely carried out in livestock animals, especially cattle, because of the large benefits to production. Females have been used to enlarge the scale of farming for livestock production, so the demand for female offspring is increasing (Seidel Jr, 2007). H-Y antibody is used in a cytotoxic reaction to reduce the amount of Y sperm and leads to increased female offspring. The study of gene mapping in mice showed that the gene on the Y chromosome is involved in male development (Gubbay *et al*., 1990). Furthermore, study of the sex-reversed (Sxr) region on the Y chromosome showed that H-Y antigen is produced by the gene that is located on the Y chromosome and that the Sxr region is involved in the production of H-Y antigen (Roberts *et al*., 1988). Therefore, many researchers have investigated the protein that is produced by males for embryo sexing or embryo identification. H-Y antigen plays an important role in the sexual development of vertebrate animals and study of evolutionary conservation in H-Y antigen (Wachtel *et al*., 1975) showed that H-Y antigen was found in heterogametic sex of almost every male amphibian, female avian and male mammalian cell (Wachtel *et al*., 1974, Wachtel *et al*., 1975). H-Y antigen is mostly found in the brain, lymphocyte, testis and sperm which are mainly located on the acrosomal cap of the sperm head (Koo *et al*., 1973). Moreover, H-Y antigen can be found in embryo cells of mouse (Shelton and Goldberg, 1984), murine (Ramalho *et al*., 2004), pig (White *et al*., 1987), and cattle (Gardon *et al*., 2004). Polyclonal antibodies and monoclonal antibodies are powerful tools to manipulate the sex ratio in cattle; they are extensively produced by using splenocytes or leukocytes as a source of antigen to obtain antibodies against H-Y antigen (Hings and Billingham, 1984; Booman *et al*., 1989; Andonian *et al*., 2001). This present study uses the sperm of White Lamphun cattle as a source of antigen because it is a native cattle breed in northern Thailand and the advantages are its high temperature resistance and ability to utilize low quality food. Therefore, the aim was to produce a monoclonal antibody against male specific antigen in White Lamphun cattle.

MATERIALS AND METHODS

Immunization

Mice were immunized according to Booman *et al*. (1989). In brief, Balb/c female

mice (n=3) were immunized by biweekly, intraperitoneal injections of 10×10^6 cells of White Lamphun sperm. The White Lamphun sperm was evaluated, collected, frozen in liquid nitrogen and washed before each use. Sperm was washed twice in phosphate-buffered saline (PBS) with centrifugation at $100 \times g$ for 5 min after each time and a sperm count was determined using a hemocytometer. Blood samples via the tail were taken from immunized female mice and centrifuged at $100 \times g$ for 5 min to collect sera for antibody titer determination by indirect ELISA.

Detection of H-Y antibody in blood samples

H-Y antibody was detected by indirect ELISA according to the method of Crowther (1995). In brief, blood from male and female White Lamphun cattle was collected and centrifuged at $100 \times g$ for 5 min to collect white blood cells. White blood cells (2×10^4 cells per well) were coated as a source of male or female antigen by diluting in coating buffer and stored at 4°C overnight. The antigen was removed and washed three times with washing buffer. Each well was coated with 200 µL of blocking buffer (3% gelatin) and incubated for 1 hr at room temperature. The buffer was removed and washed three times with washing buffer. Each well was coated with 50 µL of blood sample diluted to 1:1,000 in PBS and incubated for 1 hr at room temperature. The buffer was removed and washed three times with washing buffer. Each well was coated with 50 µL of diluted goat anti-mouse IgG HRP 1:5000 and incubated for 1 hr at room temperature. The buffer was removed and washed three times with washing buffer. Each well was coated with 100 µL of substrate buffer (o-Phenylenediamine dihydrochloride and hydrogen peroxide) and 100 µL of stop solution (4N sulfuric acid) was added to stop the reaction when color development was detected in each well. The absorbance on each plate using an ELISA reader at 492 nm was read. The O.D. unit showed the amount of antibodies in the samples and a higher number indicated a higher amount.

Hybridoma production

The hybridoma production technique followed McMichael and Fabre (1982). In brief, 63-Ag 8.653 myeloma cells were prepared 6 d before fusion. Then, 63-Ag 8.653 myeloma cells were cultured in 10% fetal calve serum (FCS), at 37°C, with 5% CO_2 in a CO_2 incubator. A female Balb/c mouse which had the highest antibody titer was boosted intraperitoneally with 20×10^6 sperm cells 2 d before fusion. Fusion between 63-Ag 8.653 myeloma cells and splenocyte cells from the immunized Balb/c female mouse was performed as follows: an immunized female Balb/c mouse was euthanized by inhaling chloroform, the spleen was collected, sterilized with 70% alcohol, then moved into Iscove's modified Dulbecco's medium (IMDM). The spleen was flushed with a 10 mL syringe containing 5mL of IMDM and dissociated by forceps. Splenocytes were moved into 15 mL tubes, centrifuged at $100 \times g$ for 10 min, lysed with 10 mL of lysis buffer (ammonium chloride 0.83%). The splenocytes were washed with 10 mL of IMDM and counted using a hemocytometer. Myeloma cells were mixed with splenocytes in the ratio 3:1 (splenocyte:myeloma). The supernatant was removed and 50% polyethylene glycol added as a fusion solution. Cells were resuspended and washed with IMDM before adding hypoxanthine aminopetrin thymidine (HAT) medium and cultured at 37°C, with 5% CO_2 in a CO_2 incubator. After 14 d, hypoxanthine thymidine (HT) was used instead of HAT and fetal bovine serum (FBS) was used on day 21. The hybridoma cells were screened to find the positive clone that produced H-Y antibody by indirect ELISA and limited to receive monoclonal anti H-Y antibody. Clones were named after the location in the tissue culture plate.

Detection of H-Y antibody in hybridoma cells

Hybridoma supernatants were screened as described in the detection of H-Y antibody in blood samples but used 50 µL of supernatant from hybridoma clones instead of blood samples

and 50 µL of diluted goat anti-mouse IgG HRP 1:3,000 instead of diluted goat anti-mouse IgG HRP 1:5,000.

Cell limiting in hybridoma cells

The positive hybridoma cells were resuspended and transferred into 5 mL of 10% FBS in 15 mL tubes. Cells were resuspended and 2 mL of supernatant was transferred into 3 mL of 10% FBS in another 15 mL tube. Cells were resuspended and 2 mL of supernatant was transferred into 4 mL of 10% FBS in another 15 mL tube. Cells were resuspended, transferred from each tube into a 96-well tissue culture plate and cultured at 37°C with 5% CO_2 in a CO_2 incubator. After the cells were grown, the supernatants from each well were collected and detected for anti H-Y antibody titer. The hybridoma cells were subjected to repeated limiting and detection of antibody titer to receive the monoclonal cell.

Evaluation of the specificity of antibody by indirect ELISA

The specificity of antibodies was evaluated as described in the detection of H-Y antibody in blood samples but used 50, 50 and 50 (25+25) µL of supernatant from positive clones named B8, C9 and B8&C9 for the source of antibody and 50 µL of diluted goat anti-mouse IgG HRP 1:3,000 instead of diluted goat anti-mouse IgG HRP 1:5,000.

Evaluation of the specificity of antibody by Immunofluorescence microscopy

To evaluate the specificity of each antibody, the protocol was modified from Booman *et al*. (1989). The male and female white blood cells of White Lamphun cattle (10 in each group) were used as a source of male and female antigen and the follow procedure was applied. Supernatant from positive clones—B8, C9 and B8&C9—were added with 100, 100 and 100 (50+50) µL in each vial and incubated for 1 hr at 37°C with 5% CO_2 in a CO_2 incubator. Goat anti mouse IgG-FITC was diluted 1:2,000 then 100 µL was added in each vial and incubated overnight at 37°C with 5 % CO_2 in a CO_2 incubator. Samples were washed with PBS, 30 µL of PBS added in each sample and detected by fluorescence microscopy. The fluorescence percentage (H-Y positive) was evaluated in each sample to determine antibody specificity using a visual count and the percentage showed the specificity of the antibody (White *et al*., 1987; Booman *et al*., 1989; Andonian *et al*., 2001).

Statistical analysis

The antibody specificity data in white blood cells were compared using a chi-square test and differences in the data were considered significant at a test level of $P < 0.05$ (Snedecor and Cochran, 1989).

RESULTS

After immunizing Balb/c mice (n=3) to produce monoclonal antibody against Y sperm using 10×10^6 sperm from White Lamphun frozen semen as a source of antigen for 12 wk, the antibody titer against H-Y antigen was detected by indirect ELISA. The results showed that three mice produced antibody amounts against male white blood cells as a source of male antigen (H-Y antigen) and female white blood cells as a source of female antigen of 0.43 and 0.46, 0.83 and 0.63 and 0.54 and 0.52 O.D. units, respectively (O.D. at 492 nm; Figure 1).

Mouse number 2 was chosen as a source of splenocytes in the fusion process to produce monoclonal antibody against H-Y antigen because it had the highest antibody titer. After fusion, 121 hybridoma cells (27.5%) were detected, 1 positive hybridoma cell (0.83%) produced antibody against H-Y antigen called P1C2 and 2 positive hybridoma cells (1.65%) produced antibody against female antigen called P5C6 and P3E6. Cells numbered P1C2, P3E6 and P5C6 produced antibody against male and female splenocytes with a source amount of male and female antigen

of 0.55 and 0.38, 0.24 and 0.63 and 0.14 and 0.46 O.D. units, respectively. P1C2 was used in the cell limiting process, evaluated by antibody titer and received positive clones named P1C2B8 and P1C2C9. The results from the indirect enzyme-linked immunosorbent assay showed that P1C2B8, P1C2C9 and P1C2B8&C9 were specific to male and female white blood cells(10 in each group) with 1.74 ± 0.03 and 1.56 ± 0.05, 1.04 ± 0.03 and 0.88 ± 0.03 and 1.47 ± 0.07 and 1.27 ± 0.07 O.D. units, respectively (Figure 2).

The result of immunofluorescence microscopy showed that the antibodies of the P1C2B8, P1C2C9 and P1C2B8&C9 clones were specific to male and female white blood cells (10 in each group) with amounts of 92.15 ± 0.87 and 69.33 ± 0.93, 91.19 ± 0.95 and 72.95 ± 1.27 and 93.27 ± 1.25 and 68.68 ± 1.23%, respectively (Table 1) in which green fluorescence was located on the cell surface of white blood cells.

DISCUSSION

ELISA is a simple tool to detect antibody in a sample because proteins such as antibodies can attach to plastic which is used in the ELISA application (Crowther, 1995). Therefore, ELISA was used to detect antibody titer in mice, to screen hybridoma cells and to estimate the specificity of antibodies. Spleen cells have been widely used as a source of antigen for immunization in many studies (Booman *et al*., 1989; Gardon *et al*., 2004; Ramalho *et al*., 2004). The current study showed that sperm was suitable to be used as a source of antigen for immunization and that each mouse has an individual ability to respond to antigen. A long period of immunization by sperm in immunized mice was reported according

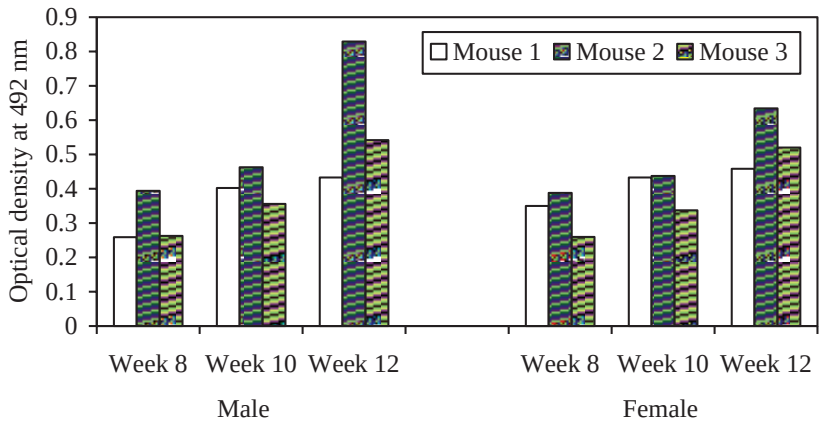

Figure 1 Antibody titer against male and female white blood cells in immunized mice detected by indirect enzyme-linked immunosorbent assay.

Table 1 Specificity of antibodies against male and female white blood cells detected by immunofluorescence microscopy.

Antibody	Fluorescence percentage in male (n=10)	Fluorescence percentage in female (n=10)	Difference in percentage (male-female)
P1C2B8	92.15±0.87[a]	69.33±0.93[b]	22.82
P1C2C9	91.19±0.95[a]	72.95±1.27[b]	18.24
P1C2B8&C9	93.27±1.25[a]	68.68±1.23[b]	24.59

[a, b] = Row means with different lower case superscripts are significantly different ($P < 0.05$).

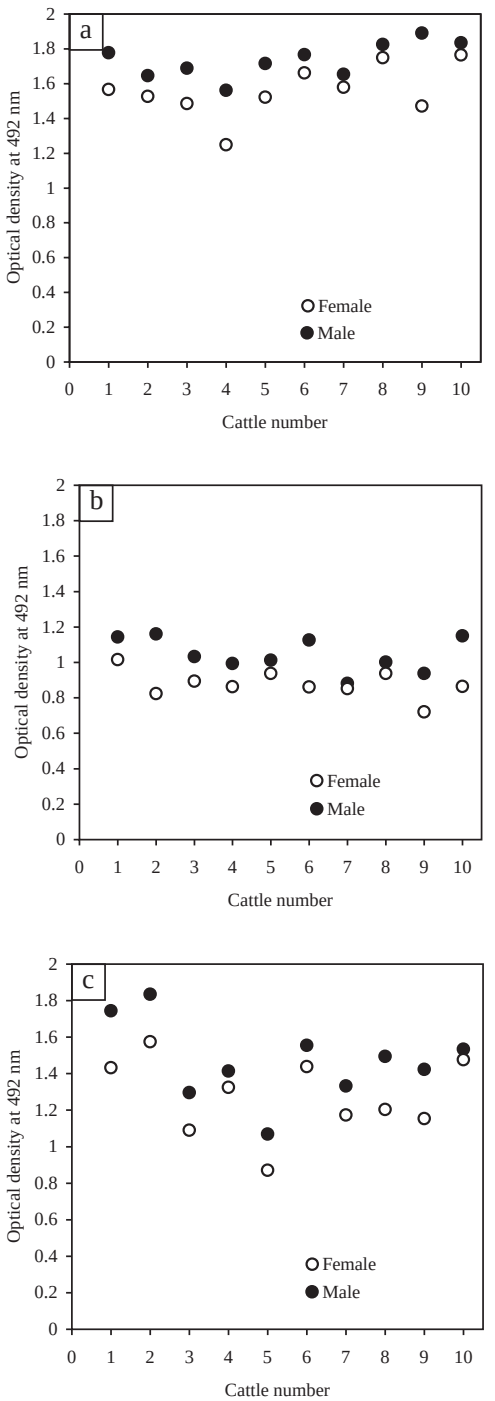

Figure 2 Specificity of the antibody: (a) P1C2B8; (b) P1C2C9; and (c) P1C2B8C9 clones against male and female white blood cells detected by indirect enzyme-linked immunosorbent assay (10 in each group).

to Booman *et al*. (1989) where spleen cells were used for immunization over a period of 9 wk. Mice produced antibody against both male and female white blood cells but mouse number 2 produced antibodies to male more than female, so mouse number 2 was chosen to be used in fusion because the opportunity to get male-specific antibody was higher. A longer period of immunization and more mice are recommended to reduce variation in antibody titer and to receive more antibody titer. In each well, many clones occurred with each clone having the ability to produce antibody differently (Abbas *et al*., 1994) because an antigen can activate the production of various antibodies (Roitt *et al*., 1996). Consequently, P1C2 was limited to obtain the monoclonal clone. The result from indirect ELISA showed that antibodies tended to be male-specific more than female-specific in every group, and multiple antibodies (P1C2B8&C9) were slightly specific to males more than single antibodies (P1C2B8, P1C2C9) were. The results from this study were different from the theory which predicts that antibody specific to H-Y antigen should be found only in male cells (Wachtel *et al.*, 1974; Wachtel *et al.*, 1975). The current study tested the hypothesis that antibody is specific to some protein which can be found on both male and female cells according to the study of calcium handling protein (Chu *et al.*, 2005) which showed the amount of $Ca_v1.2$ protein to be higher in females compared to males and indicates that the protein expression of antigen is higher in females. The results from the study showed that antibodies can bind male and female WBC but they are at higher levels in males which means the protein expression of antigen is higher in male WBC. The investigation of fluorescent dye has been used to detect male specific antigen (White *et al*., 1987) which attaches to antibody and is located in cell surface antigen (Koo *et al*., 1973) and to find the location of H-Y antigen on sperm by visual labeling. Cells were classified as fluorescent or nonfluorescent using fluorescent dye and investigated under immunofluorescence

microscopy.

CONCLUSION

The results from the immunofluorescence microscopy showed that antibodies tended to be specific to males more than females in every group ($P < 0.05$) and multiple antibodies (P1C2B8&C9) were slightly more specific to males than single antibodies which was the same as the results for the indirect ELISA. The different results of P1C2B8, P1C2C9 and P1C2B8&C9 confirmed the theory that each well has many clones and each clone has the ability to produce antibody differently. The results of this study showed that the sperm of White Lamphun cattle could be used as a source of antigen to produce monoclonal antibodies. Monoclonal antibodies were specific to males more than to females in both experiments (ELISA and immunofluorescence) and multiple antibodies were slightly specific to males more than single antibodies were and the antibodies can be used in the cytotoxic reaction to reduce Y sperm. Further study should consider the use of monoclonal antibodies in cytotoxic reactions to test the specificity of an antibody to reduce Y sperm.

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