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Large-Scale Growth of Mouse P815 Cells Expressing a Bovine Non-Classical Major Histocompatibility Complex Class I Protein Utilizing a Pitched-Blade Bioreactor

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In an attempt to express and isolate BoLA-NC3*00101, a bovine, non-classical, major histocompatibility complex (MHC) class I protein, the mouse mastocytoma cell line P815 was transfected with the NC3*00101 transgene. The transfected cells were checked for expression by flow cytometry and positive stable transfectants were sorted using a fluorescence-activated cell sorter (FACS). A large amount of purified protein is required for the generation of monoclonal antibodies in order to immunize the mice and then boost the host for several months to get highly-specific antibodies. To avoid using a large number of cell culture flasks and shorten the time required to obtain a sufficient quantity of protein, stably-transfected BoLA-NC3*00101-expressing cells were grown in a pitched-blade bioreactor. One week of culturing in the bioreactor yielded a large cell mass which was used to isolate and purify the protein.

Introduction

Transplantation-antigens were discovered as molecules responsible for acceptance or rejection of tissue grafts in mice.[1] The adaptive immune response depends on the degree of similarity among these antigens on the cells of the donor and recipient animals. The genetic region encoding the most important proteins for tissue graft compatibility is called "major histocompatibility complex." The MHC encodes two types of highly polymorphic cell surface glycoproteins, the MHC class I (MHC-I) and MHC class II (MHC-II) proteins, which present peptide antigens to T-lymphocytes. MHC-I proteins have two subsets, classical (MHC-Ia) and non-classical (MHC-Ib). Classical class I proteins are expressed on all nucleated cells in the mammalian body. Consequently, any tissue graft will express classical class I proteins and thus the recipient will react to the graft. In addition, classical class I proteins are extremely polymorphic or highly variable in the population. Therefore, tissue graft donor-recipient pairs are rarely MHC-matched.[2]

MHC-Ia proteins are usually expressed as membrane-

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bound isoforms and interact directly with the T-cell receptor. In contrast, non-classical class I proteins are expressed in specific tissues or organs and their expression may or may not be conditional. They are less polymorphic and have few variants so they do not induce a transplant rejection. MHC-Ib proteins are expressed as membrane-bound and soluble isoforms. A tissue graft from another member of the same species is known as an allogeneic transplant. A fetus is an allogeneic tissue that resides inside the maternal uterus during pregnancy.

The MHC genetic region of cattle is known as the bovine leukocyte antigen (BoLA) complex. Bovine trophoblast cells (the cells that form the outer membrane of the placenta and attach the embryo to the uterine wall) express four non-classical MHC class I genes: BoLA-NC1, -NC2, -NC3, and -NC4.[3] These non-classical class I proteins are believed to play an important role in protecting the fetal tissue graft from hostile maternal immune attack throughout pregnancy. Both non-classical and classical MHC class I proteins help protect cells against attack by natural killer (NK) cells. MHC class I-deficient cells are usually susceptible to NK cell attack and macrophage killing.[4]

To study whether the bovine non-classical class I proteins are expressed as cell surface and/or soluble isoforms, P815 (mouse mastocytoma) cells were transfected with the transgenes encoding these proteins. The mouse P815 is a transfection-competent cell line that has been used for expressing a variety of MHC class I proteins, including HLA-B27.[5] Transfected P815 cell lines have been used as immunogens to immunize mice to produce monoclonal antibodies against HLA-B27 and other HLA-B antigens.[5] In other studies, P815 cells have been transfected with equine[6, 7] and bovine MHC class I protein-encoding transgenes.[3, 8]

In preparation for this project, our transfection studies revealed that BoLA-NC3*00101 was expressed on the cell surface whereas the other three bovine MHC-Ib proteins (BoLA-NC1*00401, NC2*00102 and NC4*00201) were not expressed as cell-surface isoforms as determined with flow-cytometric analysis of transfected P815 cells. The other three MHC-Ib proteins are probably expressed as secreted isoforms. Our objective was to produce monoclonal antibodies against bovine MHC-Ib proteins and utilize the antibodies to detect soluble MHC-Ib proteins in trophoblast culture supernatants and serum from pregnant cows. Since NC3*00101 is expressed on the cell surface as a membrane-bound protein, it was possible to sort the positive and stable NC3*00101 transfectants using FACS. Scale-up in a bioreactor was used to achieve higher cell mass and isolate more protein in a shorter time than in tissue culture flasks.

To date, there are no reports of any monoclonal antibody that can differentiate the bovine NC3*00101 protein from other bovine MHC-I proteins. The availability of an NC3*00101-specific monoclonal antibody will greatly assist in determining the level of NC3*00101 protein expressed on the surface of trophoblast cells at different stages of pregnancy and the amount of protein secreted in trophoblast cultures. In order to isolate a sufficient amount of NC3*00101 protein to immunize mice for antibody production, a high number of P815 cells expressing bovine NC3*00101 protein was required. A bioreactor is an efficient, well-established and specialized, simulated biologically-active environment. Growing cells in a bioreactor yields a far greater cell mass than with culture flasks, in a shorter time, without affecting the integrity and quality of the cells or their products. Pitched-blade impellers have flat blades that are set at a 45° angle so that they provide simultaneous radial and axial flow. Combined radial and axial flow produces better mixing and promotes a high oxygen transfer rate.

Animal and plant cells often have low resistance to shear.[9] Pitched-blade impellers are low shear blades that cause less cell damage and provide gentle and smooth mixing of the cells in culture. This type of impeller is widely used with mammalian, insect, and other shear-sensitive cell-lines growing in suspension.[10] Because of these characteristics, we decided to grow the transfected P815 cells expressing NC3*00101 in a pitched-blade bioreactor.

A New Brunswick Scientific Celligen® 310 pitched-blade bioreactor assembly includes a highly sophisticated control station which possesses a touch screen interface that displays all setpoints, cascade loops, current values, and more. The compact control station includes a magnetic drive motor, integrated pumps and sensors, and provides precise control of culture conditions including: temperature, pH, dissolved oxygen (DO), and the addition of a mixture of three or four gases. This system not only has batch, fed-batch, and continuous modes for growing microbes, yeast, and fungi, but also can be adapted for growing mammalian, insect, and plant cell lines.

Materials and Methods

Cell Line

The P815 cell line is derived from a DBA/2 strain "house mouse" (*Mus musculus*) with a mastocytoma. The majority of these cells grow in suspension with a few (< 5%) cultured as attachment-dependent cells. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco® Life Technologies) with 10% bovine calf serum at 37 °C and 5% CO_2. The P815 cell line was obtained from American Type Culture Collection (ATCC).

*Transfection and FACS Sorting of Stable NC3*00101 Transfectants*

The BoLA-NC3*00101 coding sequence was amplified from complementary DNA (cDNA) using specific primers (forward primer CACCATGGGGCCGCGAACCCTC and reverse primer GATGAAGCATCACTCAGTCCCC). The pcDNA™3.1 Directional TOPO® Expression Kit (Life Technologies) was used for cloning the cDNA and expressing the protein in mammalian P815 cells. Positive clones were sequenced and a perfect NC3*00101 clone was selected for transfection. Plasmid was purified using a QIAprep Spin Miniprep Kit (QIAGEN). Lipofectamine™ 2000 (Life Technologies) was used to transfect P815 cells with the pcDNA3.1 plasmid. This vector allows for expression of a specific protein with a V5 epitope and a C-terminal polyhistidine (His-His-His-His-His-His) fusion tag. The V5 epitope permits easy detection of recombinant protein by Western blot with anti-V5 antibody and the polyhistidine tag allows rapid purification on a nickel-chelating resin which binds His-tagged proteins.

To transfect, 2×10^6 cells (1×10^6 cells/mL of DMEM) were plated per well in a six-well tissue culture plate and incubated at 37 °C, 5% CO_2. Four µg of plasmid DNA was diluted to 250 µL in DMEM and mixed gently. Lipofectamine was mixed gently before use and then 10 µL was diluted in 250 µL of DMEM and incubated for five minutes at room temperature. The diluted plasmid DNA was combined with diluted Lipofectamine® (Life Technologies), 500 µL total volume, mixed gently and incubated for 20 minutes at room temperature. A 500 µL aliquot of plasmid and Lipofectamine was added to each well containing cells and medium, and mixed gently. Cells were incubated at 37 °C, 5% CO_2 for three hours and then 5 mL of DMEM with 10% bovine calf serum was added to each well. Transfected cells were incubated for 24–48 hours prior to adding G418 Geneticin® (Life Technologies) antibiotic, 500 µg/mL, to select for stable transfectants.

After two weeks in selective media, the transfectants were screened using flow cytometry. The procedure was

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as follows: cells (1×10^6 cells/tube) were resuspended in fluorescence buffer (FB) (phosphate buffered saline [PBS] with 0.1% sodium azide, 1% bovine serum albumin [BSA]) and incubated with primary antibody for 15 minutes. The H1A monoclonal antibody was used for labeling the NC3*00101 protein. An irrelevant antibody, ColiS169A, which does not recognize the mouse P815 cells or the bovine class I protein, was used as a negative control. Both monoclonal antibodies were obtained from the WSU Monoclonal Antibody Center in Pullman, Washington USA. Cells were washed twice with FB and then incubated with a fluorescein labeled anti-mouse IgG (H+L) secondary antibody for 15 minutes. All incubations were performed at 4 °C. Cells were washed twice and fixed in PBS with 1% formaldehyde. Cells were analyzed using a BD Biosciences FACSAria™ II flow cytometer equipped with Diva software for data acquisition and analysis.

Prior to culturing the cells in the bioreactor, fluorescence-activated cell sorting was performed with the flow cytometer to isolate a subpopulation of cells expressing a high level of NC3*00101 protein. The cells were stained with the H1A monoclonal antibody as described above but were not treated with fluorescence fixative. The sorted, high-expressing cells were grown in T-75 flasks until they reached a sufficient number to seed the bioreactor.

*Culturing Sorted NC3*00101 Transfectants in the Pitched-Blade Bioreactor*

Sorted NC3*00101 cells were grown in T-75 flasks at a seeding concentration of 1×10^6 cells/mL until a sufficient number of cells were obtained. Cells were harvested from eight T-75 flasks by centrifugation at 1000 rpm (500 g) for ten minutes at room temperature. Cells were stained with trypan blue (0.4% solution) and enumerated with a Countess® Automated Cell Counter (Life Technologies). The cells used to seed the bioreactor had a viability of ≥ 95%.

A Celligen 310 stirred-tank bioreactor vessel with 5 L capacity (3.5 L working volume) was used for culturing the cells. To minimize the risk of cell shear on mouse P815 cells, a pitched-blade impeller was selected for this experiment (Figure 1). The bioreactor vessel was installed on the control station and filled with PBS before autoclaving, and the pH probe was calibrated before vessel sterilization. The sterile assembly was taken to a laminar flow hood and the PBS was replaced with 3.5 L of sterile (pre-warmed to 37 °C) DMEM supplemented with 10% bovine calf serum, 2 mM L-glutamine, and 2 mM penicillin-streptomycin using a peristaltic pump. The reactor was seeded with 2×10^5 cells/mL and the DO probe was calibrated. Cell viability was monitored using the automated cell counter. The BioFlo 310 cascades were used to control DO and pH levels. After three days of cell growth, the culture was collected into sterile bottles and cells were harvested by centrifugation at 2000 g for ten minutes at 4 °C. The process parameters (Table 1) and cascade loops are shown below (Table 2).

*Purification of NC3*00101 Protein*

Cells were kept on ice during lysis. One mL of 1× lysis buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl with 1 mM mammalian Protease Inhibitor Cocktail [Sigma-Aldrich], 1 mM phenylmethanesulfonylfluoride [PMSF]) was added per 1×10^7 cells. Cells were incubated at 4 °C for one hour with rotation. The suspension was then centrifuged at 10,000 g for 15 minutes at 4 °C to remove insoluble material and nuclei. The supernatant containing protein was collected in fresh tubes and stored at -20 °C

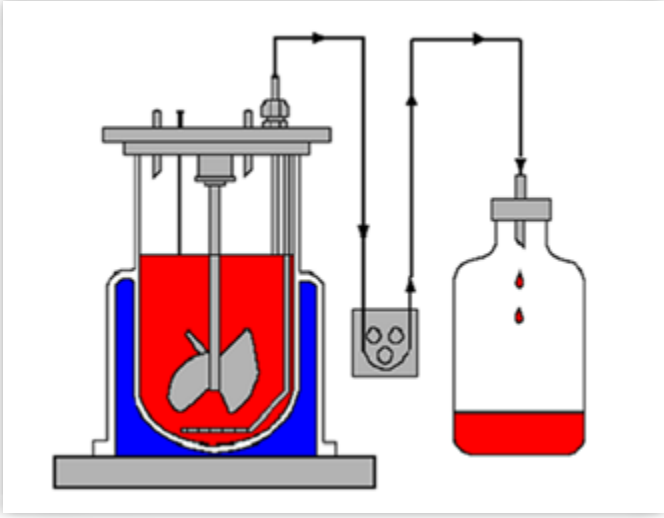

FIGURE 1. Pitched-blade bioreactor.

TABLE 1. Process parameters.

Air:	4-gas mixture (air, O_2, CO_2, N_2)
Temperature:	37°C
Gas Flow:	0.5 slpm
Agitation:	35 rpm
pH:	7.2
DO:	50%

TABLE 2. Cascade loops.

Starting Setpoint:	Starting Output:	End Setpoint:	End Output:
0.0% O_2	50% DO	100% O_2	100% DO
0.0% CO_2	25% pH	100% CO_2	100% pH

until processed for protein purification.

His GraviTrap™ columns (GE Healthcare) were used to purify the histidine-tagged protein. After loading lysate on the column, it was washed four times with a buffer containing 40 mM imidazole. Elution was performed with a buffer containing 500 mM imidazole. Purified eluates were stored at –20 °C. Eluates with strong bands on Western blots were pooled and dialyzed against phosphate buffer solution (20 mM sodium phosphate, 500 mM NaCl, pH 7.4) using 20 kilodalton (kDa) MWCO Slide-A-Lyzer dialysis cassettes (Pierce/Thermo Scientific). To prevent precipitation and to maximize the stability of proteins, 50 mM charged amino acids, L-arginine and L-glutamate were added to all of the purification buffers and dialysis buffers.[11] Dialysates were centrifuged at maximum speed for ten minutes at 4 °C and the supernatant was recovered in a fresh tube. The dialysates were concentrated using Vivaspin™ 20 concentrators with 30 kDa membranes (Vivaproducts). The concentration of each fraction was measured using a BCA protein assay kit (Pierce/Thermo Scientific).

Western Blotting

Western blots were performed with a highly sensitive Novex® WesternBreeze™ Chemiluminescent Kit (Life Technologies). 10 µL of NC3*00101 protein was mixed with 5 µL Novex® NuPAGE® LDS Sample Buffer (Life Technologies) and 5 µL deionized water and heated at 70 °C for ten minutes. 15 µL of denatured protein was loaded in each lane of a Novex® NuPAGE® 4-12% Bis-Tris Gel (Life Technologies). After 30 minutes of electrophoresis at a constant voltage of 200 V, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Life Technologies) using a Novex® XCell *SureLock*® Mini-Cell and XCell II™ Blot Module kit (Life Technologies). Transfer was performed for 80 minutes at a constant voltage of 30 V. Membranes were blocked with a buffer provided with the WesternBreeze Kit and stained with Novex® V5 Mouse mAb, AP Conjugate (Life Technologies) as per the instructions recommended by the manufacturer. Blots were evaluated by exposure of GeneMate Blue Autoradiography Film (BioExpress) for different exposure times.

Results

Flow Cytometric Analysis of Transfected Cells and Sorting

Less than 20% of newly transfected P815 cells expressed BoLA-NC3*00101 protein that could be detected by immunostaining and flow cytometry. Growth in G418 antibiotic was used to select stable transfectants. Following two weeks of selection, approximately 50% of the cells expressed immunoreactive NC3*00101 protein. Following FACS sorting with an anti-bovine MHC class I monoclonal antibody (H1A), 98.4% of the cells expressed the bovine MHC class I protein. The pre-sorting and post-sorting data are presented in Figure 2.

Growth of Cells in Pitched-Blade Bioreactor

NC3*00101-transfected cells grew with a doubling time of 24 hours. The reported doubling time for this cell line is 18–22 hours. The initial pH inconsistency was overcome by utilizing CO_2 from the pH cascade. The total number

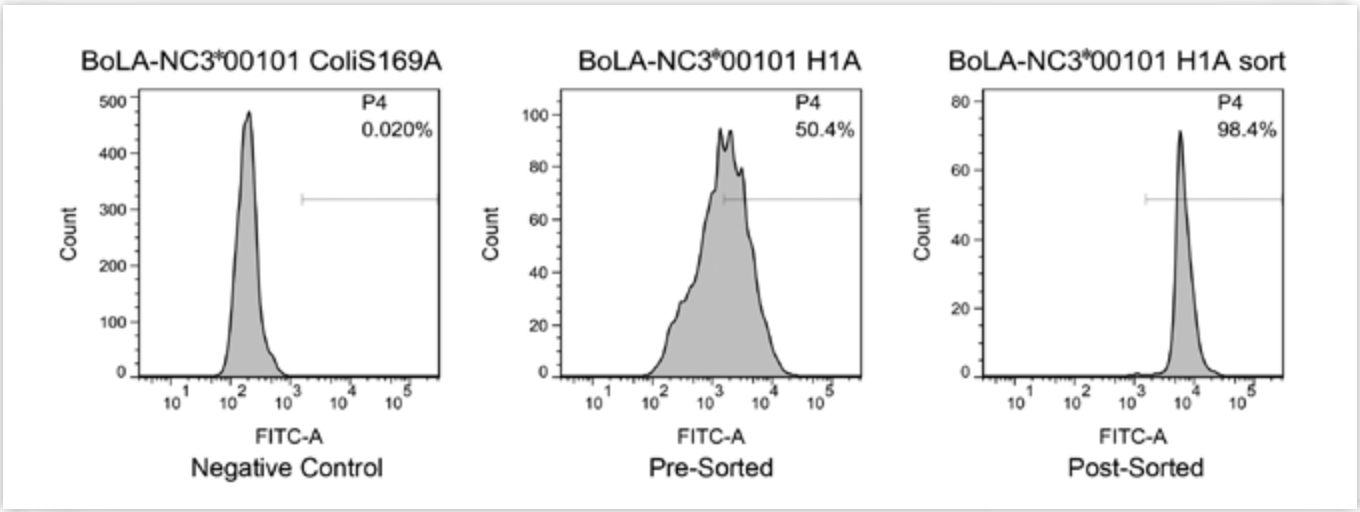

FIGURE 2. Flow cytometric analysis of murine P815 cells transfected with an expression vector encoding the bovine NC3*00101 protein. Positive, stably-transfected cells were sorted by FACS on the basis of H1A monoclonal antibody staining.

of cells harvested was 3×10^9 cells after three days of cell growth. This was an adequate number of cells for isolation of enough purified NC3*00101 protein for immunization of ten mice and subsequent screening of the hybridoma-supernatants by ELISA.

*Isolation of BoLA-NC3*00101 protein*

The concentrations measured in the crude cell lysate and purified concentrated protein sample were 1.9 mg/mL and 0.5 mg/mL, respectively. This was adequate for the downstream immunization and screening of the hybridoma supernatants using ELISA.

Western Blot

A Western blot was performed to detect the specific protein in the crude cell lysate and the purified protein sample. The blot was probed with an antibody that recognizes the V5 epitope at the C-terminal end of the protein. Non-transfected cell lysate was used as a negative control. β-galactosidase protein (120 kDa) isolated from cells transfected with the β-galactosidase control plasmid was used as a positive control. The bovine NC3*00101 class I protein was detected as a 45 kDa band in the crude lysate and the purified concentrated sample (Figure 3).

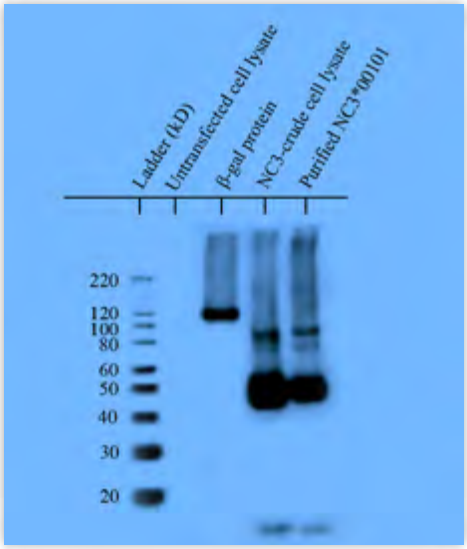

FIGURE 3. BoLA-NC3*00101 protein (45 kDa) specifically recognized by anti-V5 antibody.

Discussion

Purified BoLA-NC3*00101 protein from the bioreactor-cultured cells was used for immunization of mice and production of monoclonal antibodies. The pitched-blade bioreactor was useful in the scale-up of P815 cell growth and protein production. The product quality did not change, as measured by SDS-PAGE and subsequent detection with Western blotting. It is evident from the trends in DO and pH values that P815 cells, like CHO cells[12], utilize large amounts of oxygen for respiration in their early exponential growth phase and thus, acidify the media. Later, during the proliferation phase, the pH establishes equilibrium, followed by an elevation in pH indicating a stationary and death phase. A high pH value at 72 hours indicates high alkalinity which is possibly a marker of lack of nutrients and cell death.

Stirred-tank bioreactors have been used for culturing hybridoma cells in suspension for monoclonal antibody production.[13] Mouse P815 cells grow well in vessels with pitched-blade impellers at moderate agitation rates. Shear-sensitivity of mammalian cells has been an important issue for scaling up production but advances in technology have helped overcome this limitation.

This study resulted in successful production scale-up of murine P815 cells expressing BoLA-NC3*00101 protein using sophisticated, automated bioreactor process control machinery in batch-culture. Using the bioreactor was a significant time-saver compared to the time required for isolating the same amount of protein from cells grown in T-75 flasks. This study demonstrated the feasibility of growing FACS-sorted murine P815 cells in a large vessel without loss of protein quality or cell viability. Bioreactors are an efficient means by which difficult cells can be cultured under controlled conditions while maintaining cell health with increased productivity.

The non-classical MHC class I proteins are important immunoregulatory molecules at the maternal-fetal interface. With the generation of antibodies against these glycoproteins, it will be possible to quantify the level of

protein secretion at different stages of pregnancy. With this protein produced in the bioreactor, we were able to harvest hybridomas that secrete monoclonal antibodies to the NC3*00101 protein. These monoclonal antibodies are currently being screened by flow cytometry and ELISA. In the future, we plan to use the bioreactor to grow transfected cell lines expressing other bovine non-classical MHC class I proteins. Moreover, we will use the bioreactor to culture hybridomas secreting antibodies to BoLA-NC3*00101 and other BoLA-Ib proteins.

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