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Quantitative and sensitive detection of the SARS-CoV spike protein using bispecific monoclonal antibody-based enzyme-linked immunoassay

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The severe acute respiratory syndrome coronavirus (SARS-CoV) spike protein is known to mediate receptor interaction and immune recognition and thus it is considered as a major target for vaccine design. The spike protein plays an important role in virus entry, virus receptor interactions, and virus tropism. Sensitive diagnosis of SARS is essential for the control of the disease in humans. Recombinant SARS-CoV S1 antigen was produced and purified for the development of monoclonal and bi-specific monoclonal antibodies. The hybridomas secreting anti-S1 antibodies, F26G18 and P136.8D12, were fused respectively with the YP4 hybridoma to generate quadromas. The sandwich ELISA was formed by using F26G18 as a coating antibody and biotinylated F26G18 as a detection antibody with a detection limit of 0.037 μg/ml ($p < 0.02$). The same detection limit was found with P136.8D12 as a coating antibody and biotinylated F26G18 as a detection antibody. The sensitivity was improved (detection limit of 0.019 μg/ml), however, when using bi-specific monoclonal antibody (F157) as the detection antibody. In conclusion, the method described in this study allows sensitive detection of a recombinant SARS spike protein by sandwich ELISA with bi-specific monoclonal antibody and could be used for the diagnosis of patients suspected with SARS.

1. Introduction

The severe acute respiratory syndrome-coronavirus (SARS-CoV) is the RNA virus containing positive sense single stranded RNA (genome of 27–32 kb). The virus contains several structural proteins such as the spike (S) protein, nucleocapsid protein, membrane protein, as well as the envelope protein. All these proteins replicate in the host cell (Marra, 2003). The outbreak of SARS-CoV occurred in the Guangdong province of Southern China in 2002 and spread subsequently to 32 countries in Asia, Europe and North America. It was brought under control due to concerted world-wide efforts led by the World Health Organization (WHO). The SARS-CoV is the causative agent of SARS in humans. According to the WHO, the outbreak of SARS epidemic in 2002–2003 infected over 8400 persons and led to the death of over 900 people with a fatality rate of 9.6%. SARS-CoV-like viruses almost identical to an isolate from a patient were found in palm civet cats during the same period. Recently, the Chinese horseshoe bat has been identified as an important natural reservoir of the virus (Lau et al., 2005). In the past 10 years, the highly pathogenic SARS-CoV has been identified in humans and animals (Yang et al., 2007; Li et al., 2008; Yip et al., 2009; Yuan et al., 2010; Peiris and Poon, 2011; Balboni et al., 2012).

The vital step in preventing and controlling future epidemics is to block the transmission of infection by an effective quarantine policy which in turn depends upon early diagnosis and confirmation of the disease by laboratory tests (Poon et al., 2004). Presently, serological detection of viruses and their components by monoclonal antibodies (mAbs) is known to be a powerful method for investigating the structure and function of viral components. The production of mAbs specific for SARS-CoV aids in the study of viral pathogenesis and the development of diagnostic and therapeutic strategies. Since the development of serum antibody can take one to three weeks after SARS-CoV infection (Li et al., 2003a,b), a sensitive assay that can detect the viral nucleic acid or protein may be preferred for rapid detection of SARS-CoV infection. The profiles of antibody responses to SARS-CoV have been well established (Li et al., 2005). Reports indicate that specific viral proteins might be better markers for serological detection of infection by SARS-CoV (Tan et al., 2004; Lu et al., 2004; Berry et al., 2010).

The spike proteins of CoV are large transmembrane-glycoproteins that mediate receptor association, membrane

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fusion, and viral entry (Gallagher and Buchmeier, 2001; Bosch et al., 2003; Colman and Lawrence, 2003). They play a key role in the infection of targeted cells that possess the specific receptor (Gallagher and Buchmeier, 2001; Holmes, 2003; Hofmann et al., 2004). The cellular receptor of SARS-CoV and the receptor binding domain on S-protein have been identified (Babcock et al., 2004). Two subunits of SARS-CoV S-protein, S1 and S2, have been described (Xiao et al., 2003; Huang et al., 2006). The S1 subunit is responsible for virus binding to cellular receptor(s) and contains neutralizing epitopes, whereas the S2 subunit contains a hydrophobic fusion peptide that mediates membrane fusion following receptor association (Bosch et al., 2003). Earlier studies have shown that the receptor binding domain in the S1 region of S-protein induces neutralizing antibody, β-cell production, angiotensin-converting enzyme 2 (ACE-2) binding and virus entry (He et al., 2006). Depletion of receptor binding domain reduced significantly a humoral immune response, indicating that this domain is dominant in inducing neutralizing antibody (Lu et al., 2007). SARS-CoV S2 subunit has a highly conserved ten-residue sequence Y(V/1)KWPW(W/Y)VWL, which is rich in aromatic amino acids with 3–4 tryptophan residues. The last five residues of this region, probably from the beginning of the membrane-spanning domain, are also called the transmembrane domain (Sainz et al., 2005; Siu et al., 2008).

Information is available on mAbs for the detection of SARS-CoV (Kammila et al., 2008), however there is limited information on a sensitive detection method for SARS-CoV S-protein. Hybridomas for monoclonal antibody (mAb) and quadromas were proudced for a construction of bi-specific mAb (bsmAb) against SARS-CoV S1 antigen. The aim of this study was to evaluate a sensitive ELISA using a panel of mAbs and/or bsmAbs for the detection of SARS-CoV S1 antigen in vitro.

2. Materials and methods

2.1. Materials

Fetal bovine serum was purchased from the PAA laboratories (Etobicoke, ON, Canada) and Streptomycin–penicillin–glutamine was obtained from Gibco (Burlington, ON, Canada). Polyethylene glycol 1300–1600, HAT and Ht supplement, goat anti-mouse IgG conjugated with horseradish peroxidase (HRPO), bovine serum albumin, fluorescein isothicyanate, tetramethylrhodamine isothicyanate, horseradish peroxidase (Type IV), m-aminopheylboronic acid agarose (binding capacity to HRPO: 8–14 mg/ml of the gel), Protein G-sepharose, long chain-sulfosuccinimidyl NHS biotin, low molecular dextran sulphate, RPMI media and rabid anti-chicken IgY–HRPO were obtained from Sigma (St. Louis, MO, USA). Cell strainers for collection of spleen and streptavidin–HRPO were obtained from BD Biosciences Pharmingen (Mississauga, ON, Canada). IMAB^{TM} bags were obtained from BioVectra (Charlottetown, PE, Canada). Tetramethylbenzidine and hydrogen peroxide (H_2O_2) detecting reagent were obtained from KPL laboratories (Gaithersburg, MD, USA). Slide-A-Lyzer^{R} for dialysis was obtained form Pierce (Rockford, IL, USA). Dialysis tubing (12,000 MW cut off) was obtained from BioDesign Inc. (Carmel, NY, USA). IMAC protein purification resin was purchased form Thermo Scientific (Rockford, IL, USA). F26G18 hybridoma was kindly provided by National Microbiology Laboratory, Health Canada (Winnipeg, MB, Canada).

2.2. Preparation of SARS-CoV S1

The SARS-CoV S1 nucleotide sequence was codon optimized for prokaryotic expression and synthesized from GENEART (Burlington, ON, Canada). The optimized S1 gene was amplified by PCR and cloned in the proper reading frame in the pBM802 vector along with the His6 tag at the C-terminal for higher expression of proteins in inclusion bodies of *Escherichia coli*. The recombinant clones were analyzed by restriction digestion fragment mapping and the correct clones were selected for protein expression. Protein purification was done by IMAC chromatography from inclusion bodies. The non-glycosylated S1 protein was used to generate anti-S1 mAb and bsmAb for the development of this sensitive immunoassay.

2.3. SDS-PAGE and Western blot analyses

Purified S1 was electrophoresed on SDS-PAGE using 10% polyacrylamide gel to check the purity according to a published method (Laemmli, 1970). The protein band was electroblotted onto Hybond ECL nitrocellulose membranes (Towbin et al., 1979). The membrane was blocked with 5% skim milk in PBS containing 0.05% Tween 20 (PBS-T) for 1 h. The membrane was washed with PBS-T and incubated for 1 h with anti-SARS-CoV S1 (1 μg/ml). After washing with PBS-T, the nitrocellulose membrane was incubated with rabbit anti-S1-HRPO for 1 h. All incubations were carried out at room temperature. Finally, the membrane was washed with PBS and electrochemiluminescence detection was performed to visualize specific binding.

2.4. Production of mouse monoclonal hybridomas against SARS-CoV S1 antigen

Immunizations were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee. Briefly, 6–8-week-old female BALB/c mice (Charles River) were immunized intraperitoneally 3 times with 25 μg of S1 antigen on days 0 and 14 using complete and incomplete Freund's adjuvant, respectively, and once with 10 μg of antigen on day 28 using PBS (pH 7.3). The polyclonal antibody to the antigen was measured in mouse serum using indirect ELISA. Mice with the highest titer were spleenectomized on day 3 after the last antigen injection. Spleenocytes were fused with SP2/0 myeloma cells in a ratio of 5:1 using 50% (w/v) polyethylene glycol (PEG) (Kohler and Milstein, 1975). Five SARS-CoV anti-S1 mAbs (P135.3F3, P136.8D12, P147.4R4, P147.2R8, and P147.2R16) were developed and used subsequently for production of quadromas. The isotypes of the mAbs were determined by using isotype specific HRPO conjugated antibodies.

2.5. Cell lines for quadroma fusion

A cell line of YP4 known to secrete (IgG2a) monospecific anti-HRPO hybridoma was obtained from the late Dr. C. Milstein (Medical Research Council for Molecular Biology, Cambridge, United Kingdom). A cell line of F26G18 known to secrete monospecific anti-S1 hybridoma was kindly provided by NML (Winnipeg, MB, Canada). Anti-HRPO YP4 is a well-characterized rat hybridoma that was selected previously for drug resistance to 8-azaguanine, making it sensitive to aminopterine in HAT medium. Anti-SARS-CoV S1 F26G18 is a mouse hybridoma that can bind the receptor binding domain in S1 region of SARS CoV (Berry et al., 2010). Two cell lines of YP4 and F26G18 were chosen for developing quadromas (hybridoma × hybridoma) (Kammila et al., 2008).

2.6. Development of anti-S1/anti-HRPO quadromas

The development of anti-S1/anti-HRPO quadromas involved maintaining the two hybridoma cell lines (YP4 and F26G18, anti-HRPO and anti-S1, respectively) in the logarithmic growth phase containing RPMI medium with 10% fetal bovine serum (FBS) at 37 °C supplemented with 5% CO_2. Trypan blue staining was observed over