Vaccine 31 (2013) 3987–3994

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Development of a simple, rapid, sensitive, high-throughput luciferase reporter based microneutralization test for measurement of virus neutralizing antibodies following Respiratory Syncytial Virus vaccination and infection

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ARTICLE INFO

Article history:
Received 20 March 2013
Received in revised form 13 May 2013
Accepted 21 May 2013
Available online 4 June 2013

Keywords:
Respiratory Syncytial Virus
RSV
Neutralization
Antibody
Luciferase
Assay
Rapid
F protein
G protein
PRNT
RSV-Luc-NeuT
Vaccine
Infection

ABSTRACT

We have established a new reporter gene-based RSV neutralization test using *Renilla* luciferase. The RSV-Luciferase Neutralization Test (RSV-Luc-NeuT) is a simple, rapid, high throughput, and less labor intensive functional serological assay than the traditional RSV-PRNT, capable of measuring a broad range of anti-RSV neutralizing antibodies targeting both RSV-F and RSV-G proteins. Specificity and sensitivity of the RSV-Luc-NeuT are comparable to the RSV-PRNT. Panels of pre-vaccination and post-vaccination animal sera, monoclonal antibodies and animal polyclonal anti-RSV sera confirmed assay specificity. A panel of 60 human sera demonstrated high assay sensitivity for measurement of RSV neutralizing antibodies that strongly correlated with the RSV-PRNT titers ($R^2 = 0.864$). Neutralization in the presence of guinea pig complement (GPC) increased PRNT titers more than the RSV-Luc-NeuT neutralizing antibody titers for these human sera. This newly developed simple, high throughput, RSV-Luc-NeuT could be easily automated and applied in measurement of RSV neutralization titers in large vaccine trials.

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1. Introduction

Human Respiratory Syncytial Virus (RSV) is the leading cause of severe respiratory tract disease in the pediatric populations [1], and contributes significantly to the burden of disease in the elderly/frail and immunocompromised individuals [2]. Vaccine development was greatly hampered by the negative outcome of a clinical trial with formalin-inactivated RSV (FI-RSV) vaccine in the 1960s that did not confer long-term protection and was associated with enhanced respiratory disease and death in infants exposed to natural RSV infection following vaccination [3–5].

Neutralizing antibodies play a major role in protection against RSV infection and disease. RSV F and G proteins are the major surface viral proteins demonstrated to induce neutralizing antibodies that confer protection in animal models [6]. Furthermore, high titers of RSV specific serum neutralizing antibodies correlate with protection against RSV challenge in adult volunteers [7], and with a lower risk of RSV infection in children and the elderly [8–10]. Passively-acquired maternal anti-RSV antibodies with neutralizing activity also provide protection against lower respiratory tract infection for the first few months of life [8,9]; likewise, passively administered human immunoglobulin containing anti-RSV neutralizing antibodies protected high-risk infants from lower respiratory tract infection that led to approval of Respigam (human immunoglobulin, intravenous) for the prevention of hospitalization due to RSV disease [11,12]. Similarly, a humanized

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0264-410X/$ – see front matter. Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.vaccine.2013.05.088

anti-RSV-F monoclonal antibody with neutralizing activity was shown to provide significant protection against RSV disease in high-risk infants under 2 years of age in controlled clinical trials [13,14].

Renewed efforts are underway to develop a safe and effective RSV vaccine. Several vaccine prototypes have entered clinical trials [15,16]. The standard assay currently used to measure RSV neutralization is the plaque reduction neutralization test (RSV-PRNT) [17,18]. However, the RSV-PRNT is labor intensive, time-consuming (5–6 days), and not readily adaptable to a high throughput technology. Most importantly, the RSV PRNT is inherently variable and difficult to validate. In earlier studies in our laboratory, the level of variability for three operators was tested using RSV Lot 1 (at 1% IgG) in 27 independent RSV-PRNT assays on Hep-2 cell monolayers in the presence of 5% GPC. Plaque counts for the virus control (no antibody) for these assays ranged from 29 pfu/well to 51 pfu/well with a mean of 43 pfu/well. 50% endpoint titers ranged from 1:298 to 1:2427 (~8-fold difference between the lowest and highest titer) with a GMT ± SD of 1090 ± 430 and a coefficient of variation of 43%.

In this study, we describe the development of a simple, sensitive, fast, high-throughput reporter gene-based RSV microneutralization assay using *Renilla* luciferase-expressing RSV-A2 (rA2-Rluc) virus. We demonstrate that the RSV-Luciferase Neutralization Test (RSV-Luc-NeuT) is highly specific and sensitive for detecting both anti-F and anti-G RSV neutralizing antibodies. RSV-Luc-NeuT is more rapid (18 h) and has the potential for a higher throughput when compared with the traditional RSV-PRNT. Importantly, side-by-side comparison of monoclonal and polyclonal antibodies from animals and humans demonstrated a strong correlation between the inhibition seen using the RSV-Luc-NeuT and virus-neutralization quantified using RSV-PRNT.

2. Materials and methods

2.1. Cells and viruses

A549 cells (CCL-185) and Vero cells (CCL-81) were obtained from ATCC (Manassas, VA, USA). Recombinant RSV expressing *Renilla* luciferase (rA2-Rluc) was generated by cloning a cassette consisting of the *Renilla* luciferase (Rluc) ORF-NS1 gene end signal-NS1 gene start signal into an antigenomic cDNA (D53) of RSV at the position of the NS1 ATG. This encodes Rluc at the first position in the genome, preserving the RSV sequence from the leader through the NS1 5′ UTR (Fig. 1A). The Rluc containing D53 was then used to recover recombinant RSV in BSR-T7 cells as described [19]. Working virus stocks of rA2-Rluc were grown in A549 cells. For traditional RSV-PRNT assay, RSV strain A2, the kind gift of Drs. Robert Chanock and Brian Murphy, was plaque purified three times and then amplified in HEp-2 cell monolayers in the presence of EMEM containing 1% FBS, glutamine, penicillin, streptomycin and amphotericin B as previously described [19].

2.2. Sera and monoclonal antibodies

Control human antiserum to RSV and anti-RSV-Immune Globulin Lot 1 reference reagent were obtained from BEI Resources. IgG-depleted human serum was obtained from Sunny Labs (SCIPAQ Ltd., Sittingbourne, England). Monoclonal antibodies (MAb) 1200 (anti-RSV-F) and 1187 (anti-RSV-G) were generated and characterized as previously described [18]. Rabbit polyclonal anti-RSV G was generated by four subcutaneous immunizations with 10 μg of affinity purified protein. Pre-vaccination sera from mice, rabbits and ferrets were used as negative controls. Post-vaccination sera from rabbits and ferrets immunized with Influenza hemagglutinin proteins [25] were used to test the cross-reactivity and specificity of the assay for detection of RSV neutralizing antibodies.

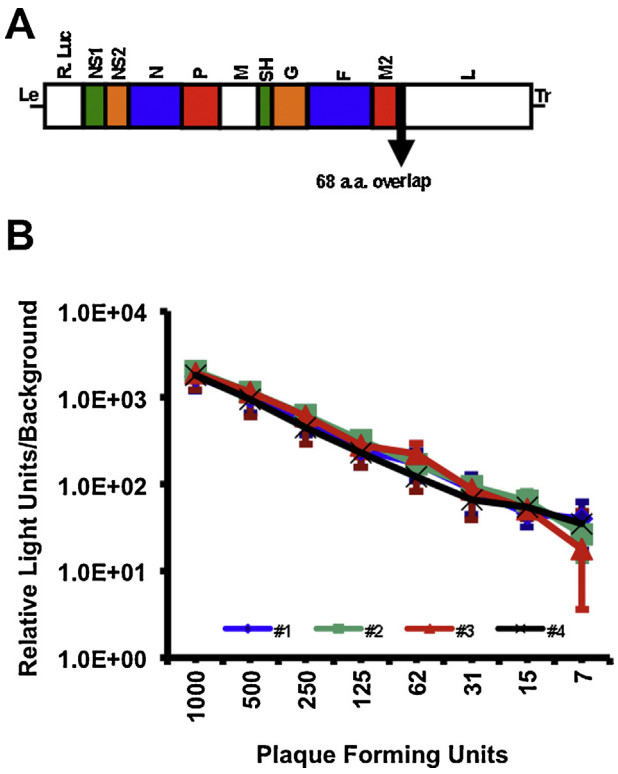

Fig. 1. (A) Schematic representation of the rA2-luc virus. The gene encoding *Renilla* luciferase is expressed from the first position in the RSV genome. (B) Virus titration. The virus was titered using Vero cells in a plaque assay to determine plaque forming units (PFUs) and a luciferase assay using A549 cells to determine the corresponding relative light units (RLU). Plaque assays were incubated for 5–7 days prior to staining. The virus dilutions were incubated for 16 h before lysing the cells and performing the luciferase assay. Error bars represent the standard deviation of the triplicate samples tested for each virus dilution in each assay. Representative data are shown for virus titrations performed in four different experiments (#1–#4), demonstrating read-out reproducibility and constant correlation between the virus titers measured in the two assays.

Human sera were obtained from a measles vaccine study originally conducted by the CDC to assess seroprevalence of measles antibody among school children in 6th–8th grade after parents gave informed consent and stored frozen in Center for Biologics Evaluation and Research (CBER) freezers since that time. Samples are anonymous and permission to test these de-identified samples for RSV neutralizing antibody was obtained from the U.S. Food and Drug Administration's Research Involving Human Subjects Committee (FDA-RIHSC).

2.3. Traditional RSV-Plaque Reduction Neutralization Test (RSV-PRNT)

The traditional RSV-PRNT was performed as previously described [17]. Briefly, serial 4-fold dilutions of heat-inactivated antibodies were mixed with virus (diluted to yield 20–50 plaques/well) containing 10% guinea pig complement (Rockland Immunochemicals; Philadelphia, PA, USA) and incubated for 1 h at 37 °C. After incubation, 100 μl of antibody–virus mixture were inoculated in duplicate onto HEp-2 cell monolayers in 24-well plates and incubated for 1 h at 37 °C. Inocula were then removed and 0.8% methylcellulose/EMEM overlay medium was added. After incubating the plates for five days at 37 °C, the overlay medium was removed and the cell monolayers were fixed with 80% methanol and immunostained with anti-RSV antibodies, as previously described [20]. The 50% endpoint titers in the PRNT assay were determined using the Kärber method.