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A flow cytometry based assay to assess RSV specific neutralizing antibody is reproducible, efficient and accurate

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Abstract

Respiratory syncytial virus (RSV) is an important cause of respiratory infection in people of all ages, and is the leading cause of hospitalization in infants. Although commercially available monoclonal antibody is available for passive prophylaxis of neonates at risk of severe disease, there is no available vaccine to prevent RSV. Measurement of neutralizing activity will be a key endpoint for vaccine evaluation. Assessment of neutralizing antibody against RSV has been limited to traditional plaque reduction, which is time consuming and inherently operator dependent and highly variable. Here, we describe a flow cytometry-based RSV-specific neutralization assay which is more rapid than traditional methods, highly sensitive and highly reproducible.

Introduction

Respiratory syncytial virus (RSV) is a pneumovirus in the family Paramyxoviridae, which also includes metapneumovirus. RSV is a major cause of infant respiratory infection, especially severe pneumonia and bronchiolitis (Glezen et al., 1981; Collins and Graham, 2008). There are three envelope proteins F, G, and SH. Both F and G are glycosylated and represent the targets of neutralizing antibodies. F-specific neutralizing antibody is known to be protective, and there is a licensed monoclonal antibody, Synagis® (Palivizumab) that is used to passively protect high risk infants from severe disease (Johnson et al., 1997). Assessment of neutralizing activity in preclinical or clinical samples has been primarily by traditional plaque reduction neutralization (PRNT) or microneutralization (Anderson et al., 1985). PRNT suffers from limited sensitivity and nonspecificity, and is prone to technician error, is tedious, labor-intensive, and is not as reproducible as newer reporter pseudovirus methods developed for other viral diseases (Mascola et al., 2002; Pierson et al., 2006;

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Martin et al., 2008). Additionally the PRNT assay is time-consuming and not easily adapted to high throughput technology. Here we describe an efficient, highly reproducible flow cytometry-based assay to detect RSV neutralization with high sensitivity and specificity.

Material and methods

Virus

Viral stocks of RSV expressing Green Fluorescent Protein (GFP) and based on the A2 strain of RSV, were prepared and maintained as previously described (Graham et al., 1988). GFP-RSV was constructed and provided by Mark Peeples and Peter Collins, as previously reported (Hallak et al., 2000). The titer of the virus stocks used for the experiments was 2.5×10^7 pfu/ml.

Cell line

HEp-2 cells were maintained in Eagle's minimal essential medium containing 10% fetal bovine serum (10% EMEM) and were supplemented with 2 mM glutamine, 10 U of penicillin G per ml, and 10 μ g of streptomycin sulfate per ml.

Antibody controls

Anti-RSV monoclonal antibody, Synagis® (palivizumab) was purchased from Medimmune, LLC (Gaithersburg, MD). Human plasma was obtained from healthy adult donors at the Vaccine Research Center clinic through an NIAID IRB approved study for blood donation at the NIH. Convalescent mouse and rabbit sera were obtained from the Viral Pathogenesis Laboratory, VRC, NIAID.

Flow cytometry neutralization assay

Antibody-mediated neutralization was measured as a function of GFP-expressing RSV infection using HEp-2 cells. GFP-RSV was added to serial four-fold dilutions (beginning with a dilution of 1:10) of (serum or antibody) in 96-well plates, which were seeded with HEp-2 cells at 5×10^4 /100 μ l per well, and incubated at 37°C for one hour. Serum concentrations ranged from 1:10 to 1:40,960. After one hour, 100 μ l of the virus/serum mixture was added to each of the wells in 96-well plates (5×10^4 cells/well). Infection was monitored as a function of GFP expression (encoded by the viral genome) at 18 hours post-infection by flow cytometry (LSR II, BD Bioscience, CA, USA). Prior to assessment by flow cytometry, cells were treated with trypsin to ensure a single-cell suspension optimal for analysis and fixed with 0.5% paraformaldehyde. Data was analyzed by curve fitting and non-linear regression (GraphPad Prism, GraphPad Software Inc., San Diego CA) to determine the percent neutralization at a given antibody concentration and the EC₅₀. Antibody concentration was adjusted to consider the final 200 μ l volume of the neutralization reaction in each well. For graphical representation raw data was “normalized” using GraphPad Prism (GraphPad Software Inc., San Diego CA) resulting in a sigmoidal dose response curve and infectivity data conversion to percent of maximal response (relative infection in percent).

Plaque reduction neutralization was performed as previously described (Graham et al., 1988). Briefly, HEp-2 cells were plated in 12 well plates in a monolayer and serial dilutions of serum were mixed with equal volumes of titered virus stock for 1 hour at 37°C. The serum dilution producing a 50% plaque reduction was calculated.

Results

The assay was optimized for consistency and sensitivity. Parameters assessed included cell culture, viral titer and infection duration. Sub confluent HEP-2 cells with a passage number between 1 and 20 were determined to be the optimal cell type. The optimized condition included a cell count of 5×10^4 per well in a 96 well culture plate, which was freshly seeded, with a control well viral infection rate of 6–12%. As the virus replication cycle and cell growth cycle affect virus infection rates, the virus infection duration is important for accuracy of results. The assay should be completed after one round of virus replication but before a secondary round of infection could potentially begin. Based on a series of time points assessed, the ideal duration of infection is 16–18 hours and this is consistent with previous studies using this construct which indicate easily detectable infectivity within 20 hours (Hallak et al., 2000).

The “percentage law” states that the amount of virus neutralized by a given concentration of antibody is constant irrespective of the total quantity of virus present so long as antibody is present in excess above the amount of virus in the assay (Andrewes, 1933; Klasse and Sattentau, 2001) (Pierson et al., 2006). Assay compliance with the percentage law provides further reassurance that the assay result reflects antibody affinity and is not related to quantity of virus present. To ascertain whether the RSV flow cytometry neutralization assay follows the “percentage law”, three dilutions of recombinant GFP-RSV at a multiplicity of infection (MOI) ranging from 0.03 to 1.5 were evaluated with serial dilutions of palivizumab with reproducible results in multiple experiments and data from a representative experiment is further described in detail (Figure 1). The EC_{50} at an MOI of 1.5, 0.15 and 0.03 was 2660, 2369 and 2372, respectively (Figure 1). The neutralization titer of palivizumab was not altered by different MOIs in the assessment of variables. Even at low virus dilutions, the EC_{50} of palivizumab is consistent when other variables like cell viability and age are fully controlled. While the assay follows the percentage law, it is noted that a set of conditions related to incubation time, cell number per well, and quality or infectivity of virus stock should be well controlled in this assay. For example, at low viral infectivity rates down to 6%, the EC_{50} of the positive control monoclonal antibody remains constant, but at viral infection rates <5% the measurement becomes unreliable. Low infection rates when using a relatively high MOI are typically caused by unhealthy cells that are at high passage number or overly confluent, or virus stock that is degraded. Therefore, to ensure consistency, we recommend control well infection rates in this assay to range from 6–12%.

Three further experiments were performed in duplicate to assess neutralization by mouse serum, rabbit serum and human plasma against RSV, at a range of MOI (Figure 2). EC_{50} of mouse serum, rabbit serum and human plasma from a representative experiment are shown to be consistent despite altered experimental conditions (Figure 2).

This flow cytometry neutralization assay has been used to assess RSV neutralizing antibody measurement in our laboratory and experimental results are consistent. Palivizumab from the same lot is used as the positive control in all experiments. To understand the degree of consistency, the EC_{50} of palivizumab from 33 experiments during a one-year period (Figure 3) were compared. Among 33 experiments, 20 were performed by operator 1 and the mean EC_{50} of palivizumab at a concentration of 1000 μ g/ml was 1733 ± 515.7 (mean $\pm$ SD), 13 were performed by operator 2 with a mean EC_{50} of 1578 ± 330.1 (mean $\pm$ SD) (Figure 3).

The reproducibility of the neutralization assay was further evaluated. A comparison of the intraoperator reproducibility was made. The neutralization titers ($\log EC_{50}$) of 16 murine serum samples were assessed by one operator, twice, in two separate experiments and the linear regression analysis shows significant correlation, $R^2 = 0.9898$, $P < 0.0001$ (95% CI of

slope is 0.9479–1.065) (Figure 3a). Next, the interoperator reproducibility was tested. Of the 16 samples tested by operator 1, 10 samples (those with sufficient volume) were available for repeat assessment by operator 2. Additionally, another 6 samples that were previously tested by operator 1 in a separate experiment were added in the interoperator comparison experiment. The linear regression analysis demonstrates that the neutralization titer ($\log EC_{50}$) between two operators correlates significantly, $R^2 = 0.9789$, $P < 0.0001$ (95% CI of slope is 0.9934–1.176) (Figure 3b). A Spearman's correlation assay also demonstrates correlation of the neutralization titer ($\log EC_{50}$) with an intraoperator correlation of $r = 0.9690$, $p < 0.0001$ (95% CI is 0.9080–0.9898) and an interoperator correlation $r = 0.9669$, $p < 0.0001$ (95% CI is 0.9019–0.9891).

A comparison of traditional PRNT to the flow-cytometry based neutralizing antibody assay was made by assessing serum from 68 cotton rats (Figure 4). The titers ranged from EC_{50} 10–3000 and a Spearman's correlation analysis shows significant correlation between the PRNT and flow cytometry based neutralization assays ($r = 0.9117$, 95% CI is 0.8584–0.9456 and $p < 0.0001$).

Discussion

Flow cytometry-based measurement of neutralizing activity for RSV is reliable and reproducible when the analysis is preformed under the optimized conditions defined here. Data derived from assessment of monoclonal antibody (palivizumab), human plasma, mouse serum and rabbit serum indicate that when performed in optimal conditions as described, the RSV flow cytometry based neutralization assay is reproducible, quantitative, and follows the “percentage law”.

The flow cytometry-based neutralization assay is a more efficient method than PRNT to evaluate RSV-specific humoral responses as it takes two days to complete (instead of 4 or 5) and up to 50 samples may easily be assessed in one experiment by a single operator. Additionally, the analysis of flow cytometry data is more efficient than manually counting plaques. The assay requires accurate cell counts and careful quality control of virus stock to ensure infectivity of the input virus. An infection rate of $\leq 5\%$ in the negative control (no antibody) invalidates the assay results and suggests a problem with the quality of the cell substrate or virus stock. Therefore, we have identified 2 criteria which should be met as a measure of quality control, the infection rate in the negative control should be $>5\%$ and the EC_{50} of the 1000 $\mu\text{g/ml}$ palivizumab positive control should ideally be within one standard deviation of the mean as established in a given laboratory setting for a given lot of antibody. The consistent EC_{50} results regardless of viral titer or infection rate indicates that the assay follows the percentage law and indicates that the assay result is a reflection of antibody neutralization and antibody affinity. To allow for this assay to be used more broadly, additional reporter viruses based on other strains of RSV, including a strain from subtype B, could be produced and assessed in a similar manner.

Assay optimization is helpful for research purposes, but for clinical product development, accurate, high-throughput assays are critical. Detection of neutralizing antibody in vaccine pre-clinical and clinical trials has historically been done utilizing the PRNT method. In recent years, viral immunologists have developed accurate, high throughput and reproducible neutralizing antibody assays, most notably in the flavivirus field (Pierson et al., 2006; Martin et al., 2007). Similar in method to the RSV assay described here, neutralizing antibody assays utilizing luciferase or GFP-expressing pseudovirus reporter systems have become more common and better standardized as new areas of viral research adopt this newer technology (Pierson et al., 2006; Sashihara et al., 2009).

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RSV monoclonal antibody Palivizumab		
MOI	EC ₅₀	95% Confidence interval
1.5	2660	2222-3185
0.15	2369	1922-2920
0.03	2372	1665-3380

Figure 1. Demonstration of reproducible neutralization of RSV-GFP by anti-RSV monoclonal antibody Palivizumab (Synagis®). EC₅₀ of Palivizumab with 95% confidence intervals under three different MOI. Sigmoidal curves of antibody dilution (x-axis) versus GFP-RSV infected cells shown as the neutralization curves at three MOI (left panel) and percent of relative infection (y-axis) in the panel to the right. Results are consistent at MOI of 1.5, 0.15 and 0.03.

	MOI	EC ₅₀	95% Confidence interval
Human plasma	2.50	160.3	98.89-260
	1.25	191.7	132.5-277.2
	0.63	343.3	265.1-449.7
	0.31	325.2	115.2-917.8
Mouse serum	2.50	850.1	749.8-963.7
	1.25	1087	915.5-1292
	0.63	1139	833.7-1556
	0.31	1185	877.8-1599
Rabbit serum	2.50	3046	2227-4167
	1.25	4373	3498-5467
	0.63	5986	4603-7785
	0.31	5968	5437-6552

Figure 2.

Consistent neutralization (EC₅₀) of human plasma, mouse serum, and rabbit serum under 4 different MOI (with 95% confidence intervals). Sigmoidal curves of antibody dilution (x-axis) versus GFP-RSV infected cells shown as neutralization curves at MOI of 2.5, 1.25, 0.63 and 0.31 (left panels) and percent of relative infection curves at MOI of 2.5, 1.25, 0.63 and 0.31 (right panels). Results are consistent among a range of MOI at 2.5, 1.25, 0.63 and 0.31.

	Operator 1	Operator 2
Number of samples	20	13
Minimum	1021	1102
25% Percentile	1245	1218
Median	1834	1649
75% Percentile	2166	1821
Maximum	2664	2148
Mean	1733	1578
Std. Deviation	515.7	330.1
Std. Error	115.3	91.54
Lower 95% CI of mean	1492	1379
Upper 95% CI of mean	1975	1778
Coefficient of variation	29.76%	20.91%

Figure 3.

Reproducibility of RSV flow cytometry neutralization assay. Figure 3a. Correlation of neutralization antibody titer ($\log EC_{50}$) by linear regression analysis of 16 mouse serum samples tested twice (on different dates) by operator 1. Figure 3b. Correlation of neutralization antibody titer ($\log EC_{50}$) of 16 mouse serum samples tested by operator 1 and operator 2 (on different dates). Results are reproducible over time and between operators. The lower panel shows a comparison of the EC_{50} of palivizumab preformed by operator 1 and operator 2 from 33 experiments during a one-year period.

Figure 4. Comparison of plaque reduction neutralization test and flow cytometry neutralization assay. 68 cotton rat serum samples were tested by plaque reduction neutralization test and flow cytometry neutralization. The linear regression analysis demonstrate significant correlation, $R^2=0.8444$, $P<0.0001$.