

Antibodies in lymphocyte supernatants can distinguish between neutralising antibodies induced by RSV vaccination and pre-existing antibodies induced by natural infection

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Keywords: Respiratory syncytial virus, antibodies, plasmablasts, antibodies in lymphocyte supernatant

Abstract

Introduction: Respiratory syncytial virus (RSV) is the single most important cause of severe respiratory illness in infants. There is no effective vaccine and the only effective treatment available is the monoclonal antibody palivizumab which reduces the risk of severe RSV disease in prematurely born infants. However, palivizumab is too costly to allow for wide implementation and thus treatment is restricted to supportive care. Despite extensive efforts to develop a vaccine, progress has been hindered by the difficulty in measuring and assessing immunological correlates of RSV vaccine efficacy in the presence of high levels of pre-existing RSV antibodies.

Methods: Here we describe a new method for measuring the functional activity of antibodies induced by vaccination distinct from pre-existing antibodies. Antibodies in lymphocyte supernatants (ALS) from the cultured peripheral blood mononuclear cells (PBMCs) of young adults who had recently been vaccinated with a novel RSV candidate vaccine were directly assayed for virus neutralising activity. An ELISA method was used to measure antibodies in nasal and serum samples and then compared with the adapted ALS based method.

Results: There was a wide background distribution of RSV-specific antibodies in serum and nasal samples that obscured vaccine-specific responses measured two weeks after vaccination. No RSV-specific antibodies were observed at baseline in ALS samples, but a clear vaccine-specific antibody response was observed in ALS seven days after the administration of each dose of vaccine. These vaccine-specific antibodies in ALS displayed functional activity *in vitro*, and quantification of this functional activity was unperturbed by pre-existing antibodies from natural exposure. The results demonstrate a promising new approach for assessing functional immune responses attributed to RSV vaccines.

Introduction

Respiratory syncytial virus (RSV) is a major cause of lower respiratory tract infection in infants, elderly adults and immunocompromised individuals.[1–8] Severe infections can cause bronchiolitis in infants[9,10] and are now recognised as a predisposing factor for the development of respiratory illness later in life.[11–14] Currently, there are no licensed vaccines or clinically effective antiviral therapies. Management of severe paediatric infections is purely supportive, and includes the use of supplemental oxygen.[15–17]

At present, there are over 60 active RSV vaccine development programmes, whose target populations range from young infants to pregnant women and elderly adults.[18–21] Development of vaccines for each group presents unique challenges.[22] The early peak incidence of severe RSV disease (~2.5 months of age) severely limits the window for paediatric intervention,[23,24] while the onset of T cell immunosenescence may hinder the development of sufficiently immunogenic vaccines for the elderly.[25]

Many of the populations in which RSV vaccine trials are typically conducted, have high levels of pre-existing, RSV-specific antibody. Many phase I RSV vaccine trials are conducted in young healthy adults, who exhibit high levels of RSV-specific antibody as a result of recurrent natural exposure[26][27]. In the case of elderly adults, although previous studies have clearly demonstrated that elderly adults with severe RSV have higher levels of neutralising antibody relative to age-matched controls[28], in general, elderly adults retain high titres of RSV-specific antibodies at levels that are comparable to young healthy adults[25,29].

In the case of infants, previous reports have shown that the peak incidence of paediatric RSV disease occurs in RSV naïve infants, within the first three months and coincides with a period in which high levels of pre-existing antibody are present[23,30–32]. In these children, the source of pre-existing antibody is vertically transferred maternal antibodies that are present at relatively high levels during neonatal life.[33]

Therefore, in the context of vaccine trials in which neonates, young or elderly adults are targeted for recruitment, distinguishing between RSV-specific antibodies that are induced by a vaccine and pre-existing antibodies induced by natural infection presents considerable difficulty. In trials of live attenuated RSV vaccines in RSV naïve infants - with high levels of maternally derived RSV antibody – despite evidence of protection against challenge with a second dose of vaccine, there was little evidence of a substantial fold increase in serum neutralising antibody following vaccination[34].

The most widely accepted correlate of protective immunity against severe RSV infection is serum neutralising antibody. In vaccine trials, this putative correlate of vaccine immunogenicity is typically characterised by representing the fold change from baseline in the titre of neutralising antibody. The accurate assessment of vaccine-induced antibodies has been complicated by the high baseline titre of pre-existing antibodies due to natural exposure to the virus.[26] Assays -such as serum-based ELISA and serum-based neutralisation tests - that are incapable of making the distinction between natural and vaccine-induced antibody may underestimate vaccine immunogenicity and negatively impact continued product development. In this project we sought to address this problem by developing an objective method of quantifying vaccine-induced antibodies distinct from pre-existing, naturally

acquired antibodies in healthy adults. Peripheral blood mononuclear cells (PBMCs) were cultured *in vitro* and antibodies produced by differentiated plasmablasts were detected in the culture supernatant. Here we show that vaccine-specific antibodies in the lymphocyte supernatants (ALS) of vaccinated individuals can be functionally characterised separately from pre-existing antibodies derived from previous antigenic exposure. The results demonstrate a promising new approach for assessing vaccine immunogenicity and will be useful in the development of RSV vaccines.

Materials and methods

Study design and population

A single centre phase I, dose-escalation, open-label clinical trial (RSV001) was conducted from April 2013 to March 2014. The trial was registered with www.clinicaltrials.gov and EudraCT (ref NCT01805921 and 2011-003589-34, respectively). A total of 42 healthy adults (aged 18-50 years) were assigned to one of four study groups and received two doses of candidate vaccine in a prime/boost combination[26]. The experimental vaccines were genetically modified viral vectored vaccines (PanAd3-RSV or MVA-RSV) that each expressed the same three RSV proteins: F, M2-1 and N. Vaccines were administered in a prime/boost regime as seen in figure 1. Written informed consent was obtained from all volunteers prior to recruitment into the study. The construction and preclinical evaluation of PanAd3-RSV and MVA-RSV, the clinical trial protocol and results of the phase I (first-in-man) safety and immunogenicity trial are published elsewhere.[26,35–37] Four time points were evaluated for each individual: i) pre-vaccination baseline, ii) one (ALS assays) or two weeks (serum and nasal antibody assays) after the first (prime) vaccine dose, iii) pre boost (just before the booster

vaccine) and iv) post boost (one and two weeks for ALS and serum/nasal antibody assays, respectively). Ethical approval for the conduct of the trial was obtained from the United Kingdom National Research Ethics Service (NRES) Berkshire (ref 13/SC/0023).

PBMC culture for isolation of antibodies in lymphocyte supernatants (ALS)

Blood samples for PBMC isolation were collected in tubes containing heparin: 400 µl of heparin per 50 ml of whole blood. PBMCs were isolated within 6 hours of sample collection. Heparinized blood was mixed with an equivalent volume of R0 (RPMI containing an antibiotic - penicillin/streptomycin - and L-glutamine, stored at 4°C) and PBMCs separated by density centrifugation using Lymphoprep (Aleris). Isolated PBMCs were cryopreserved in Recovery Cell Freezing Medium [10% dimethyl sulfoxide (DMSO) and calf serum, Invitrogen] and archived in vapour phase nitrogen. Cells were stored in vapour phase nitrogen for about 1 year prior to the analysis described in this report. Serum samples were obtained by centrifugation of whole blood collected in clotted tubes and then cryopreserved. To conduct ALS assays, cells were rapidly thawed in a 37°C water bath before re-suspension in R10 media (RPMI media containing 10% foetal calf serum, penicillin/streptomycin and L-glutamine). The cells were centrifuged at 1500rpm for 10min, after which the media was discarded and the cell pellets reconstituted in 1ml of R10. Reconstituted cells were counted using an automated cell counter (Scepter, Merck Millipore) and cell densities readjusted to 1×10^6 cells/ml by adding the appropriate volume of R10. 1ml of PBMCs (1×10^6 cells in R10) were added to the wells of a 12-well tissue culture plate (Nunc) and incubated at 37°C in a 5% CO₂ humidified incubator for 72 hours. Following this incubation period, cells were pelleted by centrifugation

(1500rpm for 10min) and the supernatants harvested and stored at -80°C until they were ready for use.

Enzyme Linked Immunosorbent Assays (ELISAs) for the detection of RSV F-specific antibodies in serum, nasal samples and ALS

Ninety six-well microtitre plates (Nunc Maxisorp) were coated overnight at 4°C with 5µg/mL recombinant F protein antigen (Sinobiological) in PBS for serum and ALS ELISAs and 20µg/mL for ELISAs utilising nasal samples (which were collected using midturbinate swabs and eluted in Copan Universal Transport Medium kit - Copan Diagnostics Inc). 200µL of blocking buffer (5% milk in PBS) was then added to each well and the plates incubated for one hour (37°C humidified incubator). For the serum ELISAs, 50µL of a 1:50 serum dilution was added to each well, while for the ALS and nasal ELISAs, undiluted samples were added to respective wells. The plates were then incubated for one hour as above and then washed three times with 200µL/well PBS. 100µL/well of 1:1000 dilutions of goat anti-human IgA (AbD Serotec) and IgG antibodies (Sigma) conjugated to horseradish peroxidase (HRP) (AbD Serotec) in blocking buffer were added to each well and incubated for one hour as above. Plates were then washed three times and developed using 100µL/well Tetramethylbenzidine substrate - TMB (Sigma-Aldrich). After 5min incubation in the dark, the reaction was terminated with 50µL/well 2M H₂SO₄. The plates were read using Biotek Elx808 absorbance microplate reader at an absorbance of 450nm.

Plaque Reduction Neutralisation Assay

Detection and quantification of neutralising antibodies in ALS samples was done by adding 25 plaque forming units (pfu) of the A2 strain of RSV to a doubling dilution series of ALS ranging from 1:2 to 1:256. 50µl of this reaction mixture (25µL A2-RSV containing 25pfu and 25µL ALS) was then added to a confluent monolayer of HEp-2 cells in 96 well tissue culture plates (Falcon). The plates were incubated for 24 hours to allow for un-neutralised virus to infect the HEp-2 cells. After the incubation period, the cells were washed once with PBS and 200µL of a carboxymethylcellulose (CMC) overlay (1% CMC in R10) was added to each well and the plates incubated for 72 hours (37°C, 5% CO₂ and 95% humidity). After this incubation the cells were washed once with PBS and fixed for 10 minutes using cold acetone/methanol (80%/20% v/v). 100µl/well of a 1:400 dilution of a mouse anti-RSV antibody (Novacastra, Leica) in PBS was added to each well and the plates incubated at 4°C for 24 hours. The plates were then washed three times with PBS, followed by the addition of a 1:1000 dilution of an HRP-conjugated goat anti-mouse IgG antibody (Biorad). After a 1 hour incubation at room temperature, plates were washed three times with PBS and developed using 3,9-aminoethylcarbazole (AEC) substrate (Sigma). The reaction was stopped after 30 minutes by washing once with PBS. The plaques were enumerated using an automated ELISpot reader (AID counter version 5). Calculation of plaque reduction neutralising antibody titres (PRNT) was done using the Spearman-Kärber method.[38]

Statistical analysis

GraphPad Prism 6 (GraphPad Software, USA) and R statistical software were used to perform data analysis. The difference between the mean antibody levels at different time points was compared using a non-parametric paired t test (Wilcoxon test).

179

180 **Results**181 *Analysis of post vaccination antibody responses in sera and nasal samples*

182 The baseline distribution of RSV-specific IgA and IgG antibody from serum and nasal samples
 183 was representative of the background natural immunity to RSV from repeated seasonal
 184 exposure. The natural humoral immunity from individual samples was highly variable (figure
 185 2). After administration of both the prime and boost vaccine there was a small but statistically
 186 significant increase in the mean levels of serum IgA and IgG two-weeks after vaccination
 187 (figure 2). Despite this, there did not appear to be a clear difference in the distribution of these
 188 antibodies before and after vaccination since the pre-vaccination baseline distributions largely
 189 overlapped with responses measured two weeks after vaccination. The same dynamics were
 190 present after administration of the booster vaccine. When the data were stratified and
 191 analysed by individual prime/boost vaccination groups, most groups did not show significant
 192 increases in antibody concentration following vaccination (supplementary figure 1).

193

194 *Analysis of responses to vaccination by measuring antibodies in lymphocyte supernatants*

195 In contrast to the baseline concentrations of serum and nasal antibody, baseline ALS antibody
 196 levels were consistently low in all groups (figure 3 & supplementary figure 2). Seven days after
 197 vaccination there were significant increases in the mean levels of RSV-specific IgA and IgG
 198 ($p < 0.0001$ and $p = 0.004$, respectively, figure 3). When the data were analysed according to
 199 individual vaccination groups, there were significant increases in RSV-specific IgA ALS antibody
 200 in all groups after prime (group 1 $p = 0.04$, group 2 $p = 0.003$, group 3 $p = 0.04$, group 4 $p = 0.04$),
 201 and in all but one group (group 2 - PanAd3-RSV IM/PanAd3-RSV IM) after boost (group 1

p=0.02, group 3 p=0.003, group 4 p=0.008, supplementary figure 2). Analysis of the IgG response in ALS showed a significant increase in two of the four groups following prime (group 2 p=0.007 and group 4 p=0.03) and in all but one group (group 2 - PanAd3-RSV IM/ PanAd3-RSV IM) following boost (group 1 p=0.04, group 3 p<0.0001, group 4 p=0.001, supplementary figure 2). This pattern of response contrasted sharply from the responses to vaccination measured in nasal samples where a significant change in nasal IgG was only seen in group 2 following priming vaccine (p=0.009) and a significant nasal IgA change was only observed in group 2 following booster dose of the vaccine (p=0.049, supplementary figure 1c and d).

Vaccine immunogenicity was also assessed by expressing the magnitude of antibody responses as fold changes in antibody level following vaccination (figure 4). In total, two volunteers seroconverted – i.e. showed a four-fold or greater increase – in serum IgA following priming vaccine, while none of the volunteers in the other groups achieved this serological threshold following the priming and booster vaccines (figure 4). Analysis of mucosal responses to vaccination showed that only two volunteers had a greater than four-fold increase in RSV-specific nasal IgG after priming dose of vaccine and one volunteer mounted a four-fold rise in nasal IgA following priming vaccine (figure 4). In contrast, when antibody responses to vaccination were evaluated in ALS, a large number of volunteers exhibited four-fold increases in RSV-specific IgG and IgA after both the priming and booster doses of vaccine. In a large proportion of volunteers RSV-specific IgA and IgG levels measured seven days after vaccination were over 10 fold higher than their respective baseline levels. In a smaller number of volunteers, increases in the post-vaccination levels of RSV IgG and IgA that were over 30-fold higher than baseline levels were observed (figure 4).

225

226 Analysis of the neutralising activity of ALS antibodies

227 To assess if ALS antibodies from vaccinated volunteers had functional activity, a modified
228 plaque reduction neutralisation assay was developed (see materials and methods section) and
229 used to determine the titre of RSV-specific neutralising antibodies in ALS. As shown in figure
230 5a, there was clear evidence that antibodies in the lymphocyte supernatant of a vaccinated
231 volunteer were capable of mediating *in-vitro* neutralisation of RSV. Lower ALS dilutions
232 exhibited potent neutralising activity, while at higher dilutions this neutralising effect was
233 reduced in proportion to the extent of dilution (figure 5a). Using this assay, ALS neutralising
234 activity was quantified in a subset of 10 volunteers from group 1 (PanAd3-RSV IM/MVA-RSV
235 IM). Baseline neutralising antibody titres prior to the priming and booster doses of vaccine
236 were measured and compared with neutralising antibody titres seven days after each vaccine.
237 Relative to pre-vaccination levels, there was a statistically significant 2.6-fold increase in ALS
238 plaque reduction neutralising antibody titre (PRNT) seven days after the priming vaccination
239 (4.3 GMT vs 11.3 GMT; $p=0.027$). However, the mean neutralising antibody response seven
240 days after administration of the booster vaccine did not significantly differ from pre-
241 vaccination levels measured prior to the administration of the booster vaccine (figure 5b).

242

243 **Discussion**

244 In this paper, we show for the first time that RSV vaccine-induced neutralising antibodies can
245 be characterised separately from pre-existing serum and mucosal antibody by quantifying the
246 functional activity of antibodies produced by antibody secreting cells in peripheral circulation.
247 In the population of healthy young adults that was recruited into this trial, the assessment of

248 vaccine-induced antibody responses in serum and nasal secretions was obscured by the high
249 levels of pre-existing antibody, resulting from seasonal exposure to RSV. In contrast, RSV-
250 specific antibodies in the culture supernatants of antibody-secreting plasmablasts in
251 peripheral circulation, could clearly be attributed to vaccination, since these cells were not
252 present in circulation prior to vaccination. Plasmablasts are terminally differentiated antibody
253 secreting cells that are derived from recently activated B cells and typically occur in circulation
254 for a short duration following antigenic exposure and disappear shortly afterwards[39]. Due
255 to their transient kinetics, they can be considered to be markers of recent antigenic exposure
256 and can be used to distinguish between contemporary and historical exposures. In this study,
257 these cells were obtained seven days after vaccination and maintained in an *in-vitro* culture
258 in order to accumulate vaccine-specific antibodies in ALS samples. The samples were collected
259 seven days after both priming and booster doses vaccination as this time point is well
260 established to be the peak day of secondary immune responses[39–41]. In a previous study,
261 we had characterised responses of vaccine recipients using ex-vivo B cell ELISpots[26]. The
262 results of ALS ELISA responses to vaccination generally align with the results of the B-cell
263 ELISpot assays. The baseline antibody levels to RSV F prior to the priming and booster doses
264 of vaccine are low in both data sets. Similarly the anti RSV-F IgA and IgG responses to RSV-F,
265 seven days after intramuscular priming by PanAd3-RSV was significantly higher than the
266 baseline response, while the response to intranasal priming by PanAd3-RSV was
267 comparatively modest in both data sets. In both assays, the IgA and IgG responses to an
268 intramuscular boosting dose of PanAd3-RSV was significantly greater than the baseline
269 antibody level in vaccines who had been primed intranasally with PanAd3-RSV but was no
270 different from the baseline response in volunteers who had been primed intramuscularly with
271 PanAd3-RSV. Similar concordance between ex-vivo B-cell ELISpots and ALS ELISA was

observed in volunteers who were intranasally primed with PanAd3-RSV and who exhibited significant increases in IgA and IgG after intramuscular MVA boosting by both assays. Taken together these observations show that the ALS assay generally reflects the results of the more conventional ex-vivo B-cell ELISpot.

The results of the ALS-based assay further demonstrate that the high levels of baseline serum antibody that are typically observed in RSV vaccine trials of diverse target populations can be largely abrogated by evaluating vaccine-induced antibody responses using the culture supernatants of peripheral plasmablasts or ALS samples. In the data presented, the ability of the ALS-based assay to identify vaccine-specific responses was highlighted by a comparative analysis of the fold change in RSV F-specific antibody levels after vaccination. While most volunteers had only modest post-vaccination fold increases in serum and nasal antibody, most volunteers exhibited a four-fold or greater increase in the levels of ALS IgA and IgG (figure 4). Thus the recurring problem of failing to observe changes in virus-specific antibody post vaccination can be addressed by the use of ALS samples to address the influence of pre-existing antibody on measurements of vaccine-induced antibody.

The novel method presented here of assessing functional immunogenicity to an experimental RSV vaccine can be used in future vaccine trials targeting different population groups. Many target populations for RSV vaccination – neonates, pregnant women, the elderly – will typically have high levels of baseline RSV-specific antibody prior to vaccination. Previous trials that have sought to quantify functional antibody responses to experimental RSV vaccines have faced the challenge of quantifying vaccine-induced humoral immunity over a background of high levels of pre-existing antibody. For instance, a live-attenuated vaccine tested in infants

failed to induce any significant increases in antibody titres post vaccination[34]. Similarly, only 22% of young healthy adults and 16% of elderly patients who received a live-attenuated RSV vaccine developed significant antibody responses[42]. Recently, a phase I trial found that a RSV F nanoparticle vaccine induced only modest responses in a group of older adults[43].

Critically, we demonstrate that RSV-specific antibodies in ALS could be functionally assayed for neutralising activity against live RSV. As yet it is unclear whether this assay could be used to assess the extent to which vaccine candidates offer protection to future infection. This was not the aim of this study using phase I data. However, it would be interesting to see whether an ALS based approach could be used in future studies to assess protection to vaccination. Ideally, this method would be applied in large phase III trials to properly address whether this is possible. Among the potential limitations of the ALS method in the context of vaccine trials are the requirement for an additional blood draw for isolation of PBMCs for the assay. Also, being moderately labour-intensive, the assay may introduce additional complexities to trial protocols, particularly large phase III trials.

In conclusion, as the portfolio of RSV vaccine candidates emerging from the preclinical stages increases, this novel ALS-based approach can be used to provide an unambiguous estimation of a critical marker of vaccine efficacy – virus neutralising antibody- by alleviating the effect of pre-existing antibody.

Conflicts of interest

A.J.P. has previously conducted clinical trials of vaccines on behalf of Oxford University funded by GlaxoSmithKline Biologicals SA and ReiThera SRL but does not receive any personal payments from them. A.J.P. is the chair of the UK Department of Health's (DH) Joint Committee on Vaccination and Immunisation (JCVI), but the views expressed in this manuscript do not necessarily represent the views of JCVI or DH.

Acknowledgements

This study was supported and sponsored by ReiThera SRL (formerly Okairos SRL), the NIHR Oxford Biomedical Research, and salary support for C.S. and P.K. (WT 091663MA) from the Wellcome Trust.

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