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The role of non-viral antigens in the cotton rat model of respiratory syncytial virus vaccine-enhanced disease

Christine A. Shaw[a], Jean-Rene Galarneau[b], Kathryn E. Bowenkamp[b,1], Kurt A. Swanson[a], Gene A. Palmer[a], Giuseppe Palladino[a], Judit E. Markovits[b], Nicholas M. Valiante[a], Philip R. Dormitzer[a], Gillis R. Otten[a,*]

[a] Novartis Vaccines and Diagnostics, 350 Massachusetts Ave., Cambridge, MA 02139, USA
[b] Novartis Institutes for Biomedical Research, Inc., 300 Technology Square, Cambridge, MA 02139, USA

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ABSTRACT

In the 1960s, infant immunization with a formalin-inactivated respiratory syncytial virus (FI-RSV) vaccine candidate caused enhanced respiratory disease (ERD) following natural RSV infection. Because of this tragedy, intensive effort has been made to understand the root causes of how the FI-RSV vaccine induced a pathogenic response to subsequent RSV infection in vaccinees. A well-established cotton rat model of FI-RSV vaccine-enhanced disease has been used by numerous researchers to study the mechanisms of ERD. Here, we have dissected the model and found it to have significant limitations for understanding FI-RSV ERD. This view is shaped by our finding that a major driver of lung pathology is cell-culture contaminants, although FI-RSV immunization and RSV challenge serve as co-factors to exacerbate disease. Specifically, non-viral products from the vaccine and challenge preparations that are devoid of RSV give rise to alveolitis, which is considered a hallmark of FI-RSV ERD in the cotton rat model. Although FI-RSV immunization and RSV challenge promote more severe alveolitis, they also drive stronger cellular immune responses to non-viral antigens. The severity of alveolitis is associated with T cells specific for non-viral antigens more than with T cells specific for RSV. These results highlight the limitations of the cotton rat ERD model and the need for an improved animal model to evaluate the safety of RSV vaccine candidates.

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1. Introduction

Although RSV is the leading cause of lower respiratory tract illness in infants [1], safety concerns have hampered vaccine development. In the 1960s, a FI-RSV infant vaccine candidate failed to induce a protective antibody response, dramatically enhanced disease after natural RSV infection, and caused two infant deaths [2–5]. Because FI-RSV has been reported to prime for enhanced pulmonary lesions in RSV-challenged mice and cotton rats, these models have been widely used to investigate the causes of ERD and to assess risks of ERD by new RSV vaccine candidates. In mice, FI-RSV-mediated ERD is an aberrant immune-mediated event characterized by pulmonary eosinophilia and an overzealous T helper type-2 (Th2) response [6–10]. ERD in cotton rats, a species more permissive to RSV infection than mice [11,12], is typified by alveolitis and enhanced peribronchiolitis, better mirroring the histology of the fatal infant cases [13–15]. Cotton rats do not exhibit clinically overt respiratory illness or weight loss upon RSV infection.

FI-RSV immunogens and RSV challenge stocks produced by standard methods for animal studies invariably contain large quantities of non-viral, cell culture-derived proteins such as bovine serum albumin (BSA). During pre-clinical RSV vaccine development, we observed striking differences in the degree of ERD produced by RSV challenge of FI-RSV-immunized control cotton rats, depending on whether the challenge stock was crude culture medium from RSV-infected cells or semi-purified virus. This serendipitous finding, together with reports that non-viral antigens can lead to ERD-like lesions by an ill-defined mechanism [16–20], led us to dissect the contributions of RSV and non-viral antigens

Abbreviations: FI-RSV, formalin-inactivated RSV; ERD, enhanced respiratory disease; RSV, respiratory syncytial virus; Th2, T helper type 2; BSA, bovine serum albumin; FI-mock, formalin-inactivated mock; pfu, plaque forming units; FBS, fetal bovine serum; pRSV, semi-purified RSV; PBS, phosphate buffered saline; i.m., intramuscular; alum, aluminum hydroxide; i.n., intranasal; ELISA, enzyme-linked immunosobent assay; HRP, horse radish peroxidase; HI, heat inactivated; EMEM, Eagle's minimum essential medium; IFNγ, interferon gamma; IL-4, interleukin 4; nt, not tested; SEM, standard error of the mean; ns, not significant.

* Corresponding author. Tel.: +1 617 871 5711; fax: +1 617 871 8759.
E-mail address: gillis.otten@novartis.com (G.R. Otten).
[1] Present address: Gilead Sciences, Inc., 333 Lakeside Drive, Foster City, CA 94404, USA.

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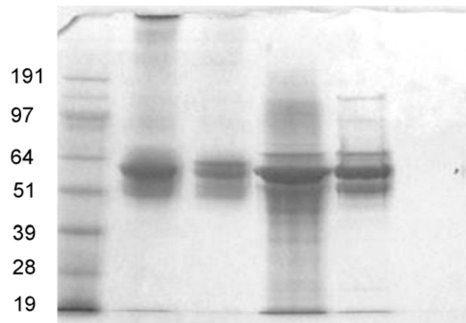

material:	FI-mock	FI-RSV	mock	RSV	pRSV
total protein (μg):	35	7.6	49	nt	nt
BSA (μg):	5.1	1.7	14	5.8	0.0040

Fig. 1. Vaccination and challenge materials contain non-viral proteins. Coomassie-stained SDS-PAGE gel of vaccine and challenge materials. One dose equivalent of each vaccine stock (4 μL FI-mock or 4 μL of FI-RSV) or challenge stock (28 μL of mock, 1×10^5 pfu equal to 28 μL of crude RSV, or 1×10^5 pfu of semi-purified RSV [pRSV]) was loaded per lane. Molecular weight ladder (kDa) is in lane 1. BSA and total protein content of one dose equivalent of the materials are indicated, nt, not tested.

to the etiology of ERD in the cotton rat model. Here, we demonstrate that cell-culture contaminants in vaccine and challenge virus preparations are dominant factors driving pulmonary lesions in this model. The severity of these lesions was associated with the magnitude of the non-viral antigen-specific T cell response, which was exacerbated in the presence of RSV antigens. These findings suggest that caution should be used when extrapolating results from this model to assess the safety of human vaccine candidates.

2. Materials and methods

2.1. Vaccines and viruses

FI-RSV and FI-mock were produced by Virion Systems, Inc., by inactivating clarified cell-culture medium from subgroup A RSV strain Long-infected HEp-2 cells (titer of 1.3×10^6 plaque-forming units [pfu]/mL) or mock-infected HEp-2 cells (each grown with 1% fetal bovine serum [FBS]), with a 1:4000 dilution of formalin for 72 h at 37 °C. A recombinant, histidine-tagged, fusion peptide-deleted RSV F protein (subgroup A RSV strain A2 sequence) ectodomain was produced in an insect cell-expression system and purified using nickel affinity and size exclusion chromatography [21]. Crude RSV (3.6×10^6 pfu/mL) and mock challenge stocks were prepared by clarifying cell-culture medium from RSV Long-infected or mock-infected HEp-2 cells (grown with 2.5% FBS). Semi-purified RSV (pRSV, 1×10^8 pfu/mL) was generated by pelleting a similarly prepared stock of crude RSV through 30% sucrose (30,000 × g for 2 h) and resuspending the pellet in phosphate-buffered saline (PBS) with 25% sucrose. Total protein and BSA concentrations in vaccine and challenge materials were determined by Bradford assay (Bio-Rad) and BSA capture enzyme-linked immunosorbent assay (ELISA) (Sygnus Technologies), and these materials were also analyzed by Coomassie-stained SDS-PAGE gel (Fig. 1).

2.2. Cotton rats, vaccinations, and infections

Female cotton rats (*Sigmodon hispidis*) were obtained from Harlan Laboratories and housed under specific pathogen-free conditions. All experiments were approved and performed according to Novartis Institutional Animal Care and Use Committee guidelines. Animals were immunized twice intramuscularly (i.m.) with a 1:25 dilution of FI-RSV or FI-mock, 5 μg of native or denatured F subunit antigen (denatured by boiling in 4 M urea followed by dialysis) adsorbed to 200 μg of aluminum hydroxide (alum), or 5 μg of native F subunit antigen/alum plus a 1:25 dilution of FI-mock or an equivalent total protein dose of untreated mock. Animals were inoculated intranasally (i.n.) with crude or semi-purified RSV (1×10^5 pfu, unless otherwise indicated), untreated mock material (diluted in the same manner as crude RSV), or a combination of semi-purified RSV and mock. All vaccines and challenge inocula were brought to 100 μL with PBS. Animals were anesthetized with isoflurane prior to i.n. inoculation and retro-orbital blood collection.

2.3. Antibody assays

Plates (96-well, Nunc) were coated overnight at 4 °C with 1 μg/mL of F protein in PBS, blocked for 1 h at 37 °C with SuperBlock Blocking Buffer in PBS (Thermo Fisher Scientific, Inc.), and then incubated with 5-fold serial dilutions of sera in PBS with 0.1% Tween-20 and 5% goat serum for 2 h at 37 °C. Bound antibody was detected with horse radish peroxidase (HRP)-conjugated chicken anti-cotton rat IgG (Immunology Consultants Laboratory, Inc., 1030-05) for 1 h at 37 °C, before addition of TMB peroxidase substrate solution (Kirkegaard & Perry Laboratories, Inc.). Titers were defined as the reciprocal serum dilution at approximately $OD_{450\,nm} = 0.5$ (normalized to a standard included on every plate). If a sample's titer was below the first dilution tested, 1:25, it was assigned a titer of 5. F-specific IgG avidity was determined by incubating antigen-bound serum with 6 M urea in PBS at room temperature for 15 min before adding detection antibody. Avidity index = (titer with urea/titer without urea) × 100.

2.4. Neutralization assay

Two-fold serial dilutions of heat-inactivated (HI) sera were preincubated with approximately 115 pfu of RSV Long in PBS with 5% HI-FBS for 2 h at 37 °C/5% CO_2 prior to inoculation of HEp-2 cells in 96-well plates. The inoculum was removed after 2 h and cells were overlaid with 0.75% methyl cellulose/Eagle's minimum essential medium (EMEM)/5% HI-FBS and incubated for 40–46 h. Cells were then fixed with 10% neutral-buffered formalin and permeabilized with 0.5% saponin, and infectious foci were stained and visualized with a cocktail of mouse anti-RSV F and anti-nucleoprotein antibodies (AbD Serotec, MCA490 and MCA491G), followed by HRP-conjugated goat anti-mouse IgG (Southern Biotech, 1032-05) and TrueBlue Peroxidase Substrate. Foci were counted using a CTL Immunospot S5 UV Analyzer. The neutralization titer was defined as the reciprocal of the highest interpolated serum dilution producing a 60% reduction in foci, relative to virus-control wells without serum. If a sample's titer was below the first serum dilution tested, 1:20, it was assigned a titer of 10.

2.5. Viral load

Lungs were homogenized, clarified, and titered by plaque assay on HEp-2 cells in 12-well plates. Cells were inoculated with serial dilutions of lung homogenate in PBS with 5% HI-FBS for 2 h at 37 °C/5% CO_2; inoculum was removed; and cells were overlaid with 1.25% SeaPlaque agarose (Lonza) in EMEM and 5% HI-FBS, and further incubated for 4 days. Cells were then stained with neutral red and plaques were counted 1 day later. If the viral load for a sample was below the assay limit of detection (~200 pfu/g lung), it was assigned a value of 100 pfu/g lung.

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