Vaccine-enhanced respiratory syncytial virus disease in cotton rats following immunization with Lot 100 or a newly prepared reference vaccine

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A formalin-inactivated respiratory syncytial virus vaccine was used to immunize infants in the mid-1960s; when these children later were naturally infected by the virus they developed markedly accentuated disease, and two died. For the present work, a new batch of vaccine was prepared using the original formula. Administration of either the old or new vaccines resulted in enhanced lesions in immunized cotton rats subsequently challenged with live virus, although administration of the vaccine reduced virus replication by 90%. Animals primed with formalin-inactivated virus and challenged developed markedly accentuated lesions of the same type as in animals undergoing primary or secondary infection. In addition, the animals with the vaccine-enhanced disease developed alveolitis and interstitial pneumonitis, which appear to be specific markers for the vaccine enhancement. The newly prepared vaccine appears suitable as a reference standard for studying the mechanism of vaccine-enhanced disease caused by this virus. Additionally, we reviewed the lesions in the lungs of the two humans who died with the vaccine-enhanced disease in 1967, and found that they were similar to, but more severe than those seen in the cotton rats.

Introduction

Four decades of effort have yet to produce an effective and safe vaccine against respiratory syncytial virus (RSV). The first clinical trials of a candidate vaccine ended abruptly when it became apparent that recipients of the vaccine were up to 16-fold more likely to be hospitalized from RSV disease than were control vaccinees (Chin et al., 1969; Fulginiti et al., 1969; Kapikian et al., 1969; Kim et al., 1969). The disastrous results of those trials continue to cast a dark shadow over the field of RSV vaccinology nearly four decades later, to the extent that no candidate non-replicating RSV vaccine has since been allowed to progress into clinical trials among immunologically naive infants.

A major impediment to demonstrating the safety of a candidate vaccine has been a lack of understanding of the mechanisms by which the earlier vaccine, a formalin-inactivated preparation designated Lot 100 (Pfizer), elicited an atypical immune response leading to enhanced disease. A desirable reference standard would be an animal model of vaccine-enhanced disease against which the safety profile of new candidate vaccines could be contrasted. Our earlier report (Prince et al., 1986) described the pulmonary pathology in cotton rats immunized with Lot 100, and then challenged intranasally with live RSV. A major limitation of that paper, however, was that Lot 100 at that time was over 20 years old and in very limited supply, insufficient to be a reference reagent.

In order to allow the elucidation of an animal model that would parallel Lot 100-enhanced disease, Wyeth–Lederle Vaccines prepared a new reference vaccine [I-RSV-01; hereafter designated formalin-inactivated RSV (FI-RSV)] using the original Lot 100 protocol. In addition, they prepared a control formulation (PL-RSV-01) consisting of uninfected cell culture inactivated and processed in an identical manner, hereafter designated formalin-inactivated Mock (FI-Mock). Characterization of RSV disease in cotton rats immunized with these formulations, as well as the original Lot 100 and an appropriate control formulation, forms the basis of the current report. Additionally, we had the opportunity to compare the histology
of the lung lesions of the two human RSV vaccine-enhanced deaths with the lung lesions in the experimental cotton rats.

Methods

- **Animals.** Inbred young adult cotton rats (*Sigmodon hispidus*) of both sexes were obtained from our breeding colony. Animals were housed in large polycarbonate cages and fed a diet of standard rodent chow and water.

- **Vaccine formulations.** Primary African green monkey kidney cells were obtained from M. A. Bioproducts (Rockville, MD, USA) and grown (37 °C, 5% CO₂) in Dulbecco’s Modified Eagle’s Medium with Earle’s balanced salt solution. The medium was supplemented with 10% heat-inactivated foetal bovine serum (HyClone); 2 mM L-glutamine and 10 mM HEPES buffer.

  The Bernett strain of RSV (originally used in the Pfizer Lot 100 vaccine) was obtained from R. Michael Hendry (California Department of Health). Dr Hendry passaged the virus three times in HEp-2 cells after receiving the strain from Robert Chanock (National Institutes of Health). Prior to use in the present studies, the Bernett virus stocks were adapted to monkey kidney cells by ten serial passages.

  Monkey kidney cells were infected at a multiplicity of 0.02 when they were approximately 90% confluent by allowing virus to adsorb for 2 h (37 °C) with rocking. Thereafter, the monolayers were washed once with medium without serum and cultured without serum until a typical cytopathic effect was detected in > 80% of the cells. The culture media (including cell debris) was harvested from the flasks, clarified by low speed centrifugation (1000 × g, 10 min, 4 °C), and filtered using a Millipore SH filter (5 µm). Formalin was added to the filtrate at a final dilution of 1:4000 and incubated (37 °C) for 3 days with stirring. The formalin-inactivated culture medium was then added to sterile tubes (Ultra-clear, 38 × 102 mm, Beckman) and ultracentrifuged (62000 × g, max., 32000 × g, centrifuged 62000 × g, max., 32000 × g, for 30 min). The resulting pellets were resuspended to 1/25th of the original volume in Basal Medium Eagle without supplements. The vaccine was then adsorbed (overnight, room temperature) to aluminium hydroxide (4 mg/ml). The compound powder was pelleted by centrifugation (10000 × g, 10 min) and resuspended to 1/4th volume in Basal Medium Eagle containing 200 units/ml each of neomycin, streptomycin and polymyxin B, and a 1:40000 final dilution of benzethonium chloride. This procedure resulted in a final vaccine that was concentrated 10-fold and contained 16 mg/ml alum.

  Lot 100, produced in the mid-1960s by Pfizer Inc. for the National Institutes of Health under contract PH43-63-582, was generously supplied by Hyun-Wha Kim, now retired from Children’s National Medical Center in Washington, DC, who was one of the participants in the clinical trials in 1965–1967. Dr Kim also provided Lot 23, a formalin-inactivated parainfluenza virus type 1 trial vaccine prepared in parallel with Lot 100 by Pfizer under the same contract. Lot 100 and Lot 23 have been maintained under refrigeration but not frozen since the time of their manufacture.

- **Challenge virus and assay of virus.** The Long strain (group A) of RSV was obtained from the ATCC. Virus stocks were prepared in HEp-2 cells and contained approximately 10⁶ p.f.u./ml. Viral titres in stocks and in organ homogenates were determined by plaque assay on HEp-2 cells (Prince et al., 1978).

- **Assay of neutralizing antibody.** Serum samples were assayed individually using a plaque-reduction neutralization assay with a 60% endpoint (Prince et al., 1978).

- **Histologic analysis.** Lungs were inflated intratracheally with 10% neutral buffered formalin in order to maintain pulmonary architecture. Following paraffin embedding, 4 µm sections were cut, and then stained with haematoxylin and eosin. Four parameters of pulmonary inflammatory changes were scored in each lung section: peribronchiolitis (inflammatory cells, primarily lymphocytes, surrounding a bronchiole), bronchitis (neutrophils within the bronchial epithelium), alveolitis (inflammatory cells within alveolar spaces) and interstitial pneumonitis (increased thickness of alveolar walls associated with inflammatory cells). Each of these parameters was scored separately for each histologic section. Prior to scoring, all of the slides were examined to determine the range of pathology for each of the four parameters. The maximum for each lesion was assigned a value of 100. The slides were then randomized, read blindly and scored for each parameter as a percentage of the maximum. Although each parameter was scored using the same scale, the scores are relative and valid only for comparing the same parameter in different sections, and not for comparing different parameters within a section.

- **Experimental design.** Three sets of experiments were performed. The first was designed to determine the dilution of vaccine that would cause maximum disease enhancement, as our earlier work (Murphy et al., 1990) had shown a two-tailed dose-response. Five groups of 4 cotton rats were immunized intramuscularly with 50 µl of 5-fold serial dilutions in PBS of FI-RSV, beginning with undiluted, on days 0 and 21. A sixth group of 4 animals served as untreated controls. On day 49 all animals were challenged intranasally with 10⁵ p.f.u. of RSV/Long in a volume of 100 µl. Five days post-challenge, all animals were sacrificed by carbon dioxide inhalation. The lungs were removed from the thorax and inflated with formalin, and histologic sections were scored to determine the dilutions of FI-RSV causing maximally enhanced disease.

  Detailed evaluation of histopathology was then done in duplicate large-scale experiments consisting of eight groups of animals: FI-RSV and FI-Mock (each at dilutions of 1:25 and 1:125); Lot 100 (undiluted and 1:5); Lot 23 (undiluted); and unimmunized. Animals were bled from the retroorbital venous plexus, and then immunized intramuscularly with 50 µl of the indicated formulation on days 0 and 21 or left unimmunized. On day 49 all groups were bled, and then challenged intranasally with 10⁴ p.f.u. of RSV/Long in a volume of 100 µl. On days 1, 2, 3, 4, 5, 7 and 10 post-challenge, groups of animals (8–10 per time-point per formulation except for Lot 23, where the small amount of vaccine required us to limit the number to 5 per time-point) were sacrificed. Lungs were removed from the thorax with the left being homogenized for virus quantification, and the right being inflated with formalin for histopathology.

  The third experiment examined the effect of time on vaccine-enhanced disease. This was occasioned by the observation that formalin-inactivated measles vaccine caused enhanced disease (‘atypical measles’), but only several years post-vaccination (Fulginiti et al., 1967), when the protective response to the vaccine had waned to the point where reinfection resulted in enhancement. For this experiment FI-RSV was given undiluted and at a 1:5 dilution, both of which resulted in minimal disease enhancement in the first experiment, as well as 1:25. Animals were immunized on days 0 and 21, challenged 6 months later intranasally with 10⁵ p.f.u. of RSV/Long in a volume of 100 µl, and sacrificed 2, 5 and 10 days post-challenge. Lungs were removed from the thorax and divided, with one being homogenized for virus quantification, and the other being inflated with formalin for histopathology.

- **Statistical analysis.** With the exception of Lot 23-immunized animals, where the scarcity of vaccine precluded the use of larger
numbers, each time-point for each formulation consisted of 8 to 10 animals. For Lot 23, there were 5 animals per time-point. Geometric means (viral titres), arithmetic means (histologic parameters) and standard errors were calculated for each time-point, and differences among groups were evaluated with Student's t-test of summary data.

Relevance to Lot 100-enhanced disease in humans. In an effort to relate our findings to the enhanced disease seen in the clinical trials of Lot 100 during the 1960s, we examined the published report of the Center having the only fatalities (Kim et al., 1969), the two autopsy reports from those fatalities, and recuts of the autopsy lung tissue. The autopsy reports and lung recuts were generously made available to us by Children’s National Medical Center, Washington, DC.

Results

Dose optimization

Fivefold dilutions of FI-RSV, from undiluted to 1:625, were used to determine the doses that would give maximum enhancement of pulmonary disease upon subsequent RSV infection. All dilutions of FI-RSV resulted in alveolitis, the primary histologic marker of enhancement (Prince et al., 1999), which was absent in RSV-infected but unimmunized control animals in the same experiment (Fig. 1). Maximum alveolitis was seen with dilutions of 1:25 and 1:125, which were used subsequently to characterize, in detail, vaccine-induced enhancement.

Characterization of enhanced disease

Virus replication and histopathology incident to FI-RSV immunization were compared to animals receiving FI-Mock at the same dilutions. In addition, animals immunized with Lot 100 were compared to those receiving Lot 23, a parallel formulation containing formalin-inactivated human parainfluenza virus type 1 rather than RSV. All groups were also compared to unimmunized animals that were challenged intranasally with RSV.

FI-RSV at either dilution (1:25 and 1:125) reduced viral titres by up to 90% when compared either to FI-Mock or unimmunized animals (Fig. 2, P < 0.01 for days 2–5). In spite of these reductions in viral titre, FI-RSV resulted in elevated levels of all four histopathologic parameters. Two of these, peribronchiolitis and bronchitis, were seen in all groups of animals, but were significantly higher in the FI-RSV groups (P < 0.02 for peribronchiolitis on days 3–5, Fig. 2). This Figure does not show bronchitis, which followed a similar pattern.

The other two, alveolitis and interstitial pneumonitis, were seen almost entirely in the FI-RSV groups (P < 0.02 for alveolitis on days 4 and 5, Fig. 2). This Figure does not show interstitial pneumonitis, which followed a similar pattern.

A similar trend was seen with Lot 100, although the differences were less striking, probably due to loss of potency after cold storage for over 35 years. Again, viral titres were reduced by up to 90% compared to Lot 23 or unimmunized animals (Fig. 3, P < 0.02 for days 3–5), yet histopathologic lesions were increased. The increases in peribronchiolitis were significant compared to unimmunized animals (P < 0.05 on days 2–4) but not in comparison to Lot 23 animals, whose numbers were small in consequence of the limited amount of Lot 23 available. Similarly, alveolitis in Lot 100 animals was significantly greater than in unimmunized animals, in which none was seen at any time-point (P < 0.02 on days 3, 4, 7 and 10), but did not reach statistical significance when compared to Lot 23 animals.

None of the animals receiving any of the formalin-inactivated RSV or control preparations developed measurable serum neutralizing antibody (1:20) at either time-point post-immunization (21 or 49 days). Animals infected intranasally with RSV but not immunized with one of the formulations developed an average titre of 1:74 at day 21, and 1:67 at day 49 post-infection.

Effect of time post-immunization

Children immunized with a formalin-inactivated measles vaccine, similar in its formulation to Lot 100, were protected from measles for several years, but gradually became susceptible to infection leading to enhanced disease, known as ‘atypical measles’ (Fulginiti et al., 1967). By contrast, vaccine-enhanced RSV disease was reported in the first winter post-immunization, but not thereafter. We examined this phenomenon in cotton rats, delaying challenge for 6 months after initial immunization. Histopathology scores for all parameters were reduced at all three vaccine dosages (undiluted, 1:5 and
Fig. 2. The top panel shows pulmonary viral titres, expressed as log_{10} p.f.u. per gram at days 1, 2, 3, 4, 5, 7 and 10 post-challenge. Each bar represents the geometric mean of 8–10 animals, with standard error. Values in FI-RSV-treated animals that are significantly reduced (P < 0.05) from FI-Mock and unimmunized groups are highlighted by an asterisk. The middle and bottom panels show scoring of peribronchiolitis and alveolitis. Each bar represents the arithmetic mean of 8–10 animals, with standard error. Values in FI-RSV-treated animals that are significantly increased (P < 0.05) from FI-Mock and unimmunized groups are highlighted by an asterisk.

1:25) compared to animals challenged 42 days post-immunization as in earlier experiments (data not shown), a result consistent with the Lot 100 clinical trials.

**Review of human autopsy materials**

The first autopsy described the lumina of the large bronchi being ‘filled with large numbers of neutrophils, macrophages, and giant cells’. The bronchial epithelium was ‘infiltrated with neutrophils and mononuclear cells’. The lumina of the smaller bronchi and bronchioles were ‘filled with neutrophils, lymphocytes and in some areas increased numbers of eosinophils’ (emphasis added). The epithelium of the small bronchi and bronchioles was ‘infiltrated with neutrophils and mononuclear cells’, with ‘numerous aggregates of neutrophils within vacuoles of the bronchial epithelium’. Alveoli generally contained ‘scattered infiltrates of neutrophils and macrophages’, while those of the right lower lobe contained ‘many neutrophils and mononuclear cells’. In other words, the large majority of granulocytes described in the autopsy report were neutrophils, with a single mention of eosinophils ‘in some areas’. Our own examination of the lung sections confirmed the above description. We estimate that the bronchial exudate contains 50% neutrophils, 30% macrophages, 20% lymphocytes and a few, occasionally fused, epithelial cells. The peribronchial inflammation was composed of 10% neutrophils, 10% macrophages and 80% lymphocytes. In both the bronchial lumen and peribronchial inflammation Giemsa-stained sections contained 1–2% eosinophils. We are at a loss to explain why eosinophils, by far the minor component of the granulocyte population, were
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Fig. 3. As in Fig. 2, with different formulations. Viral titres in Lot 100-treated animals that are significantly reduced ($P < 0.05$) from Lot 23 and unimmunized groups are highlighted by an asterisk. Values in Lot 100-treated animals that are significantly increased ($P < 0.05$) from unimmunized group are highlighted by an asterisk.

mentioned in the published paper, while the intense neutrophilia was not.

Similarly, the report of the second autopsy attested to the preponderance of neutrophils. The lumina of the large bronchi contained 'a mixture of neutrophils and mononuclear cells', and the bronchial epithelium was 'infiltrated with neutrophils and mononuclear cells'. The walls of the larger bronchi contained 'a dense infiltrate of neutrophils and lymphocytes' and 'in some areas... increased numbers of eosinophils' (emphasis added). The lumina of the smaller bronchi and bronchioles contained 'large numbers of neutrophils and mononuclear cells' and 'in some areas increased numbers of eosinophils' (emphasis added). Finally, the walls of the smaller bronchi and bronchioles were 'infiltrated with neutrophils and mononuclear cells'. Our review of the second case demonstrated less cellular bronchial exudate than the first case, with a similar distribution of cell types. The peribronchial cellular infiltrate was quite similar to that of the first case. Additionally, some lymphoid aggregates were present in the interstitial areas. Giemsa stains showed about 2% of the cells in the bronchial lumen and peribronchial inflammation to be eosinophils. A comparison of lung tissue from the second autopsy with that from an FI-RSV-immunized cotton rat is shown in Fig. 4.

It is possible that the presence of the neutrophils was attributed to bacterial infection of the lungs, as *Klebsiella* was cultured from the lungs of the first autopsy, and *E. coli* from the second. However, we stained recuts from both autopsies with Gram stain and found no visible bacteria in either case, suggesting that the reported positive cultures may have been due to small numbers of bacteria that were insignificant in what
was primarily a viral pneumonia. Since pulmonary neutrophilia was not, at that time, considered part of the pathogenesis of RSV pneumonia (Aherne et al., 1970), one may understand why the neutrophilia might have been overlooked as the major immunopathologic process.

**Discussion**

The lasting legacy of the Lot 100 clinical trials is an overriding concern over the safety of any non-replicating RSV vaccine candidate, and in the intervening years none has advanced to clinical trials in immunologically naïve infants, the primary target population. Characterization of vaccine-enhanced disease in an animal model that could be used generally as a standard would be advantageous, but as yet there has been no consensus as to what the animal model should be, or what markers of enhanced disease would best serve to evaluate new vaccines.

The current studies were initiated at the request of the Division of Microbiology and Infectious Diseases, National Institute of Allergy and Infectious Diseases, with the goal of defining a reference vaccine and animal model. Since the amounts of Lot 100 still available were inadequate – and long outdated, having been manufactured nearly four decades earlier – Wyeth–Lederle Vaccines agreed to prepare a new lot of FI-RSV following the protocol used for Lot 100. In addition, in order to address the issue of whether non-viral components of the vaccine might be implicated in enhanced disease, they made, in parallel, a formulation, FI-Mock, that differed from FI-RSV only in the absence of virus.

**Dose response**

In our initial evaluation of FI-RSV we found a two-tailed dose response, with either too much or too little vaccine giving submaximum enhancement. This was consistent with our
earlier report (Murphy et al., 1990) which used a simpler version of formalin-inactivated RSV. On the basis of this initial experiment we chose two dilutions, 1:25 and 1:125, which gave maximally enhanced disease in cotton rats, and examined their effect in large-scale experiments of more than 500 animals.

**Relationship of viral titre to histopathology**

FI-RSV and Lot 100 vaccines were effective in lowering viral titres by as much as 90% in cotton rats, clearly a positive outcome if viral titre is the only marker of vaccine efficacy considered. In spite of the substantial reduction in viral infectivity, animals immunized with these formulations had significantly higher levels of pulmonary histopathology in each of the four parameters that we scored. The inverse relationship between viral titre and enhanced disease points to allergic sensitization rather than the same pathogenesis as primary RSV disease as some have suggested (McIntosh & Fishaut, 1980).

**Specificity of enhanced disease**

Several models of enhanced disease in animals immunized with various FI-RSV preparations have been reported. These have included cotton rats (Prince et al., 1986, 1999), African green monkeys (Kakuk et al., 1993), mice (Waris et al., 1996) and calves (Gershwin et al., 1998). In contrast to these reports, however, other investigators have described experimental conditions that, in their hands, did not produce virus-specific enhancement (Piedra et al., 1989a, b, 1993; Vaux-Peretz et al., 1992).

The current report, whose histopathologic studies involved more than 500 animals, employed two formulations of formalin-inactivated RSV and two appropriate controls. In the case of FI-RSV the control was a mock-infected cell culture that was processed in parallel with FI-RSV, and used in animals at the same dilutions. In the case of Lot 100 the control was a formalin-inactivated parainfluenza virus type 1 that was processed in parallel with Lot 100. In both instances there was a clear-cut difference between RSV and control formulations in alveolitis, the histopathologic parameter that we previously reported as the primary marker of vaccine-enhanced disease (Prince et al., 1986, 1999). The minor amount of alveolitis seen in animals immunized with FI-Mock (3 of 20 animals on day 7) appears to be due to the fact that these animals were sensitized to cellular antigens and challenged with similar cellular antigens, analogous to the prototypical report of experimental pulmonary alveolitis (Johnson & Ward, 1974).

Although peribronchiolitis was also more pronounced in FI-RSV and Lot 100 groups than in control animals, we were not able to differentiate the groups on the basis of tissue morphology. It is possible that the development of reagents specific for cotton rat T-cell subsets, which is in progress in our laboratory, will eventually allow peribronchiolitis also to be used as a marker of enhanced disease. In the meantime, alveolitis remains the best histologic marker for enhancement in the cotton rat, and is most pronounced 4 and 5 days post-challenge.

The chief histologic findings of enhanced disease in the cotton rat that we have focused on in this report – alveolitis consisting primarily of neutrophils, and peribronchiolitis consisting primarily of lymphocytes – match those of the two Lot 100 fatalities. The fact that peribronchiolitis is a general finding, whereas alveolitis is restricted to FI-RSV and Lot 100, underscores the importance of alveolitis as a histologic marker of vaccine-enhanced disease. The published report commented only briefly on the histopathologic findings of the autopsies, citing ‘peribronchiolar monocytic infiltration with some excess in eosinophils’ (Kim et al., 1969). Unfortunately, this description does not accurately reflect the complete findings of the autopsies, and its mention of eosinophils and lack of mention of neutrophils have led many to conclude that eosinophilia is a primary marker of vaccine-enhanced RSV disease (e.g. Bembridge et al., 1998; Johnson et al., 1998; Sparer et al., 1998; Tebbey et al., 1998). Vaccine-enhanced RSV disease in two other mammalian species, African green monkeys (Kakuk et al., 1993) and calves (Gershwin et al., 1998), is also characterized by neutrophilic alveolitis, without eosinophils. By contrast, enhanced disease in mice is not accompanied by neutrophils (Connors et al., 1992), and pulmonary eosinophilia, while predominant in some strains of mice, is absent in others (Hussell et al., 1998).

We believe that the pathogenesis of vaccine-enhanced RSV disease is complex and multifactorial, and a comprehensive understanding of it will require studies of chemical mediators (cytokines and chemokines) as well as histopathology. Furthermore, studies of histopathology described in the current report are foundational rather than complete, and the development of reagents to detect cell-surface markers in the cotton rat, particularly of T lymphocytes, will shed additional light. We are in the midst of a comprehensive effort to develop reagents for detecting and quantifying cotton rat cytokines, chemokines and cell-surface markers. Genes that have been completely cloned and sequenced include CD18, IFN-α, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 p35, IL-12 p40, IL-18, IP-10, IFN-1, MCP-5 homologue, KC, MHC class I (three alleles), MIP-1, MIP-2, RANTES and TNF-α. Partially cloned genes include CD4, CD8α, CD11b, Cox-1, Cox-2, ICSBP, IRF-2, JE, TGF-β1 and TNF-β. Preparation of reagents for the assay of transcription and translation of these genes is proceeding in collaboration with R&D Systems, Inc. We estimate that utilization of these reagents to extend the observations of the current report will occur within 2 years.

On the basis of this and prior reports, we suggest that the cotton rat provides a model of vaccine-enhanced RSV disease which reproduces the neutrophilic alveolitis characteristic of enhancement in humans. This model may provide insight into the efficacy and safety of candidate RSV vaccines.
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References


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