Increased replication of respiratory syncytial virus (RSV) in pulmonary infiltrates is associated with enhanced histopathological disease in bonnet monkeys (Macaca radiata) pre-immunized with a formalin-inactivated RSV vaccine

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The pathology of respiratory syncytial virus (RSV) disease in bonnet monkeys parallels findings with human RSV disease. RSV-infected animals pre-immunized with a formalin-inactivated (FI) RSV vaccine develop inflammation in peribronchiolar, perivascular, interstitial and intra-alveolar sites with lung inflammation scores significantly higher than animals with a primary RSV infection and those pre-immunized with an FI-Vero cell control vaccine (P = 0.05). Animals previously infected and re-exposed to RSV had significantly lower alveolar, interstitial and total lung inflammation scores than in primary infection (P = 0.05). Immunization with two intra-muscular doses of 0·5 ml of the FI-RSV vaccine administered 21 days apart resulted in little serum-neutralizing and ELISA antibody, low levels of secretory IgA and a low lymphocyte proliferative response that was significantly lower than the response observed in animals that were previously infected with live RSV. Higher RSV virus titres were detected in the lungs and lung lavage fluid of monkeys immunized with the FI-RSV vaccine than in those with a primary infection (P = 0.001). RSV was detected by in situ hybridization in pulmonary inflammatory infiltrates, where the single most abundant infiltrating cellular species was macrophages, so it may be these cells that support the enhanced virus replication that contributes to the enhanced pulmonary pathology of FI-RSV immunization.

Introduction

Respiratory syncytial virus (RSV) is the most important virus respiratory pathogen of infancy. This virus infects nearly all children in their first year of life and 0·5–2·0% of the infected infants require hospitalization for this respiratory infection (Collins et al., 1996). Despite the enormous burden of human disease created by this virus, there remains no effective vaccine to protect from and diminish the effects of infection with RSV. This deficiency is due in part to a prior adverse human experience with a formalin-inactivated (FI) RSV vaccine tested in children in the 1960s. Also there remains an incomplete understanding of the biology of enhanced disease after FI-RSV immunization. The FI-RSV vaccine, while immunogenic, was abandoned because subsequent RSV infections tragically caused more severe lower respiratory disease in the FI-RSV-immunized seronegative infants as compared to infants immunized only with trivalent parainfluenza vaccine (Fulginiti et al., 1969). The mechanism by which FI-RSV vaccine enhances RSV disease in humans requires elucidation before newer vaccines can be given to seronegative infants. The mechanism for enhanced disease must be investigated in a non-human animal model that will mimic the enhanced disease that occurs in children.

A wealth of information about RSV pathogenesis has been derived from the mouse model. RSV-specific T helper (Th) lymphocytes are implicated in the inflammatory response to RSV that occurs in naive and vaccine-primed mice and humans (Graham, 1995; Openshaw et al., 1988). Primary and secondary infections of mice with RSV induce a predominant Th1 lymphocyte response detected in spleen (Openshaw et al., 1988).
1988), lung (Graham et al., 1993) and bronchoalveolar lavage (BAL) specimens (Waris et al., 1996). Conversely, in animals pre-immunized with FI-RSV vaccine high levels of interleukin-4 (IL-4) mRNA are detected, suggesting that a Th2 lymphocyte response contributes to the enhanced lung pathology (Graham et al., 1993). In mice, depletion of CD4 cells (Conners et al., 1992) or depletion of Th2 cytokines IL-4 and IL-10 (Conners et al., 1994; Tang & Graham, 1994; Waris et al., 1996) reduces the severity of enhanced disease. Transfer of CD4 Th2-type cells secreting IL-4 into RSV-infected mice is associated with loss of body weight and with increased infiltration of eosinophils in BAL (Alwan et al., 1994). These mouse experiments suggest that primary infection with RSV elicits a Th1 response, while an RSV infection following FI-RSV vaccine induces a Th2 response and enhanced pathology.

The pathogenesis of enhanced disease has not been fully studied in the primate models. The current work shows that FI-RSV vaccine-induced enhanced disease in bonnet monkeys (Macaca radiata) is associated with virus replication in the inflammatory cells around blood vessels.

**Methods**

**Monkeys.** Healthy, feral, infant and juvenile bonnet monkeys (Macaca radiata), weighing between 1000 and 2500 g, were quarantined for 1 month. Tuberculin-negative and RSV- and simian T-lymphotropic virus-1-seronegative monkeys were used in the experiments performed at the Primate Facility at the Christian Medical College Hospital (CMCH), Vellore, India. This facility has established Animal Care requirements and is under the direction of a licensed veterinarian. This study was approved by the Animal Welfare Committee of the CMCH and by the Animal Care and Use Committee of the University of Colorado Health Sciences Center. The study groups are described in Table 1.

**Vaccine preparation.** Vero cells were grown in 150 cm² plates in Dulbecco’s Modified Eagle’s Medium (DMEM) with 5% foetal bovine serum (FBS) and antibiotics at 37 °C in 5% CO₂. At 80% confluence, the plates were infected with Burnett strain RSV at an m.o.i. of 0:01 and maintained in Basal Medium Eagle (BME) without FBS. When the cells showed approximately 90% cytopathic effect (CPE), the medium was removed and the cells were harvested by scraping and centrifuged at 450 g for 10 min. Formalin was added to the supernatant to a final concentration of 1:4000 followed by 72 h of incubation at 37 °C and centrifugation at 32000 g for 30 min. The pellet was suspended in 1/25 the original volume of BME and compounded with 4 mg/ml aluminium hydroxide (Serva, Crescent Chemical) overnight on a rocking platform at room temperature. The material was centrifuged at 120 g for 10 min and the pellet re-suspended to 1/4 volume with serum-free BME containing 200 µg/ml each of neomycin, streptomycin and polymyxin B and 1:40,000 dilution of benzethonium chloride. Uninfected Vero cells were treated similarly and used as the FI-Vero cell vaccine. All vaccines were given intra-muscularly.

**Virus stock preparation.** The RSV Long strain was selected because of its human origin. It was grown in HEP-2 cells by infecting 150 cm² plates at an m.o.i. of 0:01. When the CPE reached 50%, the medium was replaced by serum-free DMEM. When the CPE was approximately 90%, the cell monolayer was harvested with a rubber policeman into DMEM, snap-frozen in liquid nitrogen, thawed, clarified and snap-frozen in 1 ml aliquots. An aliquot of the virus stock was titrated using an enzyme-linked immuno-plaque assay, as described later.

**Infection of monkeys.** Monkeys were anaesthetized with 5 mg/kg ketamine intravenously and intubated using a 2:5 or 2:5 Fr gauge endotracheal tube. The virus was instilled into the bronchi of the left and right lungs. Infected and uninfected animals were housed in separate rooms in individual cages.

**Bronchoalveolar lavage (BAL).** Monkeys were anaesthetized with ketamine, intubated and a BAL was performed using three 5 ml/kg aliquots of normal saline through a cuffed 2:5–3 Fr gauge endotracheal tube. Lavage fluid was obtained using an 8 Fr gauge feeding tube passed through the endotracheal tube. Aliquots of aspirated BAL fluid were frozen for virus culture and antibody activity. BAL samples were collected from two monkeys from each of the groups on days 1, 2 and 3 post-inoculation (p.i.), and from all monkeys on day 7 p.i.

**Table 1. Details of the study groups**

Each of the Groups I–VI consisted of five or six monkeys, except Group V, which had only three monkeys.

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>Immune manipulation</th>
<th>Day of RSV infection (5 × 10⁶ p.f.u.)</th>
<th>Day of autopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Naive controls</td>
<td>None</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>Primary RSV</td>
<td>None</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>III</td>
<td>Tertiary RSV infection</td>
<td>2 Doses live 5 × 10⁶ p.f.u. RSV 21 days apart</td>
<td>42</td>
<td>49</td>
</tr>
<tr>
<td>IV</td>
<td>FI-RSV (high)</td>
<td>2 Doses 500 µl FI-RSV 21 days apart</td>
<td>42</td>
<td>49</td>
</tr>
<tr>
<td>V</td>
<td>FI-RSV (low)</td>
<td>2 Doses 50 µl FI-RSV 21 days apart</td>
<td>42</td>
<td>49</td>
</tr>
<tr>
<td>VI</td>
<td>FI-Vero control</td>
<td>2 Doses 500 µl FI-Vero 21 days apart</td>
<td>42</td>
<td>49</td>
</tr>
</tbody>
</table>
**Enhanced RSV replication with FI-RSV vaccine**

**Fig. 1.** Photomicrographs from the lungs of animals (H&E staining). (a) Group I, control animals showing minimal perivascular inflammation; (b) Group II, showing mild interstitial infiltration 7 days after primary RSV infection; (c) Group III, showing interstitial and submucosal infiltration in animals undergoing a tertiary RSV infection; (d) Group IV, showing severe inflammation in the peribronchovascular regions of animals receiving high dose FI-RSV vaccine; (e) Group V, with moderate perivascular inflammation with lymphoid hystiocytic cells after low dose FI-RSV vaccine; and (f) Group VI, with moderate perivascular and interstitial inflammation after FI-Vero cell vaccination. Bars indicate 100 µm.

**Collection of serum samples.** Blood samples were collected on the day of RSV inoculation and at necropsy for antibody measurements. Serum samples from the blood were frozen at $-70^\circ$C prior to assay.

**Specimen collection at necropsy.** At necropsy the chest was opened and sections of the upper and lower lobes of the left lung were placed in M4 medium (Bio Whittaker) for virus culture. Perihilar lymph...
nodes were dissected out and placed in RPMI 1640 (Gibco/BRL) with 10% pooled heat-inactivated monkey serum (RPMI-MS) for lymphocyte culture. The pulmonary artery was identified and a solution of 30% barium sulfate and 10% gelatin in PBS at 60 °C was infused at a pressure of 80 mm Hg. The left bronchus was then clamped and the right lung inflated for at least 2 h with paraformaldehyde–lysine–periodate infused into the trachea via the endotracheal tube at 40 cm pressure to fix lung tissues in an expanded state. The fixed lungs were transferred to 70% ethanol for histology and in situ hybridization.

- **Histological studies.** Peripheral and central regions of upper and lower lobes of paraformaldehyde-fixed lungs were processed and embedded in paraffin. Sections of 5 µm were stained with haematoxylin and eosin (H&E). The severity of the pulmonary inflammatory response was scored in a blinded manner according to a histopathological scheme similar to that described by Piedra et al. (1993). Four sections from the central and peripheral parts of the upper and lower right lung were scored according to the degree of inflammation in interstitium, alveoli and surrounding airways and vessels. A value of 0 (none), 1 (minimal), 2 (mild), 3 (moderate) or 4 (severe) was assigned to each histological site, and the scores were totalled for each animal. The sum of these scores was used as a total lung inflammation score. Every section was scanned entirely to assign an inflammation score with the most inflamed areas evaluated. The highest score for each parameter (peribronchiolar, perivascular, interstitial and alveolar) that involved at least 25% of each section evaluated was used.

- **Virus culture.** Two parts of each BAL sample were mixed with 1 part of M4 medium. The samples were frozen at −70 °C until they were titrated for virus. Lung samples collected at necropsy were weighed, suspended in M4 medium (Simoes et al., 1999), clarified and frozen at −70 °C until they were tested by a plaque assay using immuno-staining for the detection of virus plaques (Piedra et al., 1989).

- **In situ hybridization for the detection of RSV in lung tissue.** To localize sites of virus replication in the lung, in situ hybridization was done on all six groups of animals. RSV N gene cDNA (Johnson & Collins, 1989) (a kind gift from Peter Collins) was transcribed with SP6 RNA polymerase. An anti-sense probe was used to detect the positive-sense replicative intermediates that are present during RSV replication. RNA probe was generated from N gene cDNA cloned into a pGEM vector. Transcription was performed using 1 x Ribonuclease buffer (Promega) with 40 U RNasin (Promega) and 1 mM each of ATP, GTP, CTP, 0.5 mM UTP and 0.35 mM digoxigenin-11-UTP (Boehringer Mannheim). The optimal concentration of each probe in preliminary trials was done on all six groups of animals. RSV N gene-specific genomic RNA was present. In situ hybridization was done on sections from the lower central lobe of each monkey according to the methods described earlier (Ponnuraj et al., 1998; Rotbart et al., 1988). The specificity of in situ hybridization was checked by testing positive sections with a riboprobe derived from the 5’ non-coding region of poliovirus. To quantify in situ labelling of infiltrates, arterioles identified by intravascular barium (size 30–300 µm in diameter) with >50% circumferential labelling were examined under 100 x magnification and the radial thickness of the area with signals measured using a micrometer eyepiece. Ten measurements were made per slide and the sums of the readings were expressed as a score on a scale of 0–100.

- **Identification of cells in inflammatory infiltrates in FI-RSV-enhanced disease.** Histological sections of lungs from the animals immunized with FI-RSV vaccine and from control animals were used for...
immuno-staining to identify the specific cell types in the inflammatory infiltrates. Effective cross-reactions were identified with human cytokeratin (an epithelial cell marker), CD45 (a leukocyte common antigen marker), CD68 (a macrophage marker) and myeloperoxidase (a marker for macrophages and polymorphs). These monoclonal antibodies were obtained from Dako Corporation. Monoclonal antibody binding was detected by Vectastain ABC Elite kit (Vector Laboratories) as per the instructions provided by the manufacturer. 3,3'-diaminobenzidine was used as the substrate and the sections were counter-stained with Gill’s no. 3 haematoxylin.

- **Anti-RSV antibody assays.** Neutralizing antibody titres were performed using a micro-neutralizing assay as previously described (Simoes et al., 1999). Serum IgG and BAL fluid IgA antibodies to RSV were detected by ELISA. UV-irradiated RSV antigen and control antigen for coating ELISA plates were prepared according to a protocol previously used (Vai et al., 1985). The RSV antigen and cell culture control antigen were diluted 1:20 in PBS and incubated on Immulon II plates overnight. The wells were then blocked with 1% gelatin. Monkey sera and a parallel reference standard plate were diluted 1:10–1:10 and the BAL samples were diluted 1:1–1:10. Diluted samples were added to the antigen and control wells and incubated for 30 min. Subsequently, the wells were washed with PBS containing 0.1% Tween 20 and incubated with peroxidase-conjugated anti-isotype antibodies (Cappel, Organon Teknika; cat. #55226). The absorbance values (A) were read on a Dynatech reader and the log of the values was regressed against the log of dilution. The IgG and IgA antibody results in monkey serum and BAL samples were expressed as a percentage of the reference positive control.

- **Lymphocyte proliferation studies.** Lymphocytes were isolated from peripheral blood and peribronchial lymph noded of each monkey. The lymphocyte concentration was adjusted to be 10⁶ cells/ml in RPMI-MS and incubated at 37 °C. Lymphocytes were cultured without stimulation, or were stimulated with UV-irradiated RSV antigen. After 6 days of culture the cells were pulsed with 0.25 mCi (9.25 MBq) [methyl-³H]thyidine (ICN) for 4 h. The cultures were then harvested onto glass fibres in a 96-well harvester and the incorporated thymidine was determined with a Betaplate reader (Wallac). The mean value of triplicate experiments was determined and the preliminary analysis was done on the basis of the stimulation index (SI). A value of SI 3 was taken as evidence of a proliferative response.

- **Statistical methods.** The significance of differences between groups with respect to inflammation scores, virus replication in the lung, antibody response and lymphocyte proliferation was tested by the two-tailed Student t-test. A Chi-square for trend was used to test for differences in virus replication in the BAL samples for FI-RSV-vaccinated animals and those with primary infections. Correlation between inflammation scores and host factors was done using a correlation matrix, correcting for groups. The significance of Spearman’s rank correlation coefficient was obtained using a t-test.

## Results

### Immunization with FI-RSV vaccine elicits enhanced pulmonary pathology upon subsequent RSV infection

Inflammation in the lungs of uninfected control animals was minimal (Group I, Fig. 1a). There were moderate inflammatory changes in the lungs of monkeys with primary infection (Group II, Fig. 1b) and with tertiary infection (Group III, Fig. 1c). The animals immunized with FI-RSV vaccine at both dosages (high and low dose) showed severe inflammatory changes after RSV inoculation (Groups IV and V, Fig. 1d, e). The FI-Vero cell vaccine-immunized animals (Group VI, Fig. 1f) showed more inflammation than animals with primary infection. Animals immunized with FI-RSV (Groups IV and V) or with FI-Vero cell vaccine (Group VI) had greater total lung inflammation scores than animals with primary or tertiary infections (P = 0.01) (Fig. 2a). The differences in total lung inflammation scores reflected statistically significant differences in perivascular, peribronchial and interstitial sites (P = 0.05). There was little difference between the alveolar scores in the animals infected for the first time and those infected after the FI-RSV vaccine. Animals with tertiary RSV infection had
Fig. 4. Photomicrographs of in situ hybridization showing replicative intermediates of RSV N gene using a digoxigenin-labelled riboprobe. (a) Group I, uninfected control with no signal; (b) Group II, primary infection (arrowheads show signals in alveolar epithelial cells and arrow shows signals in inflammatory cells in perivascular site); (c) Group III, tertiary infection has minimal signals, shown by arrowheads in the perivascular area; (d) Group IV, FI-RSV full dose vaccine with profuse signals in peribronchiolar, perivascular, interstitial and alveolar sites; (e) Group V, FI-RSV low dose vaccine; and (f) Group VI, FI-Vero vaccine. Bars indicate 100 µm.

alveolar scores that were significantly lower than those of animals with primary infection or FI-RSV-immunized animals ($P = 0.02$). The characteristics of primary infection were alveolitis and interstitial pneumonitis. Animals immunized with FI-RSV had inflammation in the perivascular and peribronchial regions in addition to alveolitis and interstitial pneumonitis. Total lung inflammation scores in animals that were immunized with FI-Vero cell vaccine were higher than those found in primary infection. The interstitial and alveolar scores were not significantly different in the animals immunized with FI-Vero...
cell vaccine and the animals with primary infection. However, the FI-Vero cell vaccine group had significantly higher perivascular and peribronchiolar scores in comparison with primary infection ($P = 0.05$). These scores were significantly lower than those of the FI-RSV vaccine group.

Pulmonary inflammation in the FI-RSV-immunized RSV-infected animals was more pronounced than in animals with primary RSV infection. With enhanced disease the inflammation was located centrally around vessels and bronchioles, and peripherally in septal interstitium and alveolar spaces. The

Fig. 5. Photomicrographs of inflammatory infiltrates in animals pre-immunized with FI RSV vaccine. (a) H&E staining showing multinucleate giant cells (arrow) and eosinophils (arrowheads) along with lymphocytes; (b) cytokeratin staining; (c) staining with CD45 marker; (d) myeloperoxidase marker; (e) CD68 marker staining peribronchiolar infiltrates; (f) CD68 marker staining perivascular infiltrates. Bars indicate 100 µm.
Replication of RSV in the BAL and lung samples is increased in the FI-RSV-vaccinated animals

Virus titres in the BAL samples were high on day 1 p.i. and decreased to undetectable levels by day 3 p.i. in primary infection (Fig. 3 a). Animals with tertiary infection had undetectable levels of virus in BAL at each of these time-points. In animals immunized with FI-RSV (full dose), the titre increased with time. Virus titres remained low in animals immunized with low dose FI-RSV. Titres in animals with FI-Vero vaccine resembled those of primary infection. The full dose FI-RSV-immunized animals had about 10-fold more RSV in their lungs than animals with primary infection (Fig. 3 b) and this difference was statistically significant ($P < 0.001$). The animals given the low dose FI-RSV vaccine (Group V) had significantly more virus isolated from their lungs than animals with primary (Group II, $P = 0.001$) or tertiary (Group III, $P = 0.001$) infection. Animals given FI-Vero cell vaccine demonstrated similar levels of virus replication as seen in a primary infection. Animals undergoing tertiary infection did not shed virus in the BAL and had significantly less virus in the lung ($P = 0.01$).

In situ hybridization was used to detect the extent and site of virus replication in the lungs of the six groups of animals. The scores for the detection of positive-strand RNA using the antisense probe (Fig. 3 c) were significantly higher in the FI-RSV-immunized animals as compared to animals with primary ($P = 0.0001$) or tertiary ($P = 0.0001$) infection. The uninfected controls had no signals (Fig. 4 a). Small amounts of RSV mRNA were detected in alveolar and perivascular sites in animals with primary (Group II, Fig. 4 b) or tertiary (Group III, Fig. 4 c) infection. Extensive viral mRNA was detected in the animals immunized with FI-RSV. Distribution of viral mRNA was predominantly in interstitium, peribronchiolar and perivascular areas in the FI-RSV-immunized animals (Fig. 4 d, e). Viral mRNA was located in perivascular areas in the FI-Vero cell vaccine animals (Fig. 4 f). With the sense probe for detection of genomic RNA, only weak signals were seen in the FI-RSV-immunized animals.

Identification of inflammatory cells

Since virus replication was found in peribronchiolar and perivascular areas with maximal inflammation, attempts were made to identify the specific cell types involved. Standard H&E stain showed that the inflammatory infiltrates consisted of macrophages, polymorphs, eosinophils, lymphocytes and multinucleate giant cells (Fig. 5 a). Cytokeratin staining was negative in the inflammatory cell population, staining only alveolar respiratory lining cells and not intra-alveolar inflammatory cells or macrophages (Fig. 5 b). Good CD 45 (leucocyte common antigen) staining was identified at all sites (Fig. 5 c). Myeloperoxidase-positive cells were at all sites (Fig. 5 d). However, a large number of cells in the perivascular and

submucosa of small bronchi and bronchioles showed more of a monomorphic lymphoid infiltrate. In addition, the alveolar spaces frequently contained an increased number of mixed inflammatory cells including macrophages. Bronchial disease consisted of combined hyperplasia and metaplasia of respiratory mucosa with the inflammatory infiltrates consisting primarily of lymphocytes.

![Fig. 6. The immune response was determined in different ways. (a) Serum-neutralizing antibody. Pre- and post-infection titres were measured before and 7 days after the challenge infection. Note neutralizing antibodies only in Groups III and V. (b) IgG in serum and IgA in BAL immediately before the challenge infection. (c) Stimulation index (SI) of lymphocytes from hilar node and blood measured 7 days after the challenge infection by thymidine uptake. Bars between groups indicate statistically significant differences in SI of lymphocytes derived from hilar lymph nodes. Animals with tertiary infection had significantly greater SI than animals with primary infection. Indicates $P = 0.03$, or those immunized with FI-RSV; indicates $P = 0.01$. Animals with low dose FI-RSV vaccine had greater SI than animals with high dose FI-RSV. indicates $P = 0.005$.](image-url)

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correlation between histopathological scores and RSV titres per gram of lung tissue.

peribronchiolar inflammatory infiltrates were macrophages, as identified by CD68 staining (Fig. 5e, f).

FI-RSV vaccine elicits limited antibody and T cell responses

Little neutralizing antibody was made by animals pre-immunized with FI-RSV (full dose) or after primary infection ($P = 0.05$, Fig. 6a). Animals with tertiary RSV infection had good neutralizing antibody levels. The only group of animals that had significant amounts of IgA in BAL and serum IgG detectable by ELISA were the animals with two prior exposures to RSV (Group III, Fig. 6b).

The proliferation of lymphocytes from hilar lymph nodes in animals of Group III was significantly higher than in Group II ($P = 0.034$) or Group IV ($P = 0.01$) (Fig. 6c) animals. The SI values for Group IV animals were significantly lower compared to Group V. The proliferation of blood lymphocytes showed no difference between the groups even though there was wide variation in SI values.

Correlation between inflammation scores and virus titres

There was positive correlation between inflammation scores and virus titres. The correlation of virus titres versus total inflammation scores was $r = 0.66$ ($P = 0.0001$) (Fig. 7a). Better correlation was seen with perivascular scores versus virus titre $r = 0.79$ ($P = 0.0001$) (Fig. 7b) and peribronchial scores versus virus titres $r = 0.78$ ($P = 0.0001$) (Fig. 7c). Lower correlation was seen with interstitial scores versus virus titre $r = 0.6201$ ($P = 0.0003$) (Fig. 7d).

Discussion

Enhanced pathology following FI-RSV vaccine immunization

The studies on the bonnet monkey, *Macaca radiata*, show that there is enhanced lung pathology when the monkeys are immunized with an FI-RSV vaccine prior to an RSV infection. This monkey model of RSV disease resembles the enhanced disease that occurs in human children (Fulginiti et al., 1969), African green monkeys (Kakuk et al., 1993), mice (Connors et al., 1992; Graham, 1995; Waris et al., 1996) and cotton rats (Prince et al., 1978, 1986) with RSV infection after FI-RSV immunization. The bonnet monkey model reproduces the enhanced pathology that tragically was experienced in human children in the 1960s. This model allows for experimental immune manipulations on monkeys, which cannot be done in children. Potentially this monkey model will provide a means to allow for the development and testing of a successful protective vaccine for RSV infection.

Enhanced pulmonary pathology following FI-RSV vaccine immunization occurs in all animals at all of the evaluated pulmonary sites. Statistically significant differences are identified in total, peribronchial, perivascular, interstitial and alveolar inflammation scores between the FI-RSV vaccine-immunized group as compared to the primary infection group. These differences are greatest in peribronchial and perivascular sites, with lesser but consistent differences also present at interstitial and alveolar locations. In all cases, the inflammation in FI-RSV vaccine-immunized animals was greater than that in animals with primary infection.

While overall lung inflammation scores for the FI-Vero cell-immunized animals were not statistically different from the
scores of primary infected animals, the peribronchial and perivascular sites had scores that were significantly increased as compared to primary disease. This increased inflammation did not reach the levels seen in the FI-RSV animals, however. In BALB/c mice also mock vaccine produced histopathological changes of a lower magnitude in comparison with those immunized with the FI-RSV vaccine (Bolen et al., 2001). Immunization with FI-Vero cell vaccine has already been reported to create enhanced pulmonary pathology upon subsequent RSV infection in cotton rats (Piedra et al., 1993). The mechanism by which an FI-Vero cell vaccine creates enhanced pathology is not known. One possibility is that the small amounts of foetal calf serum proteins in the Vero cell vaccine sensitize the vaccine recipients and that repeat exposure to these proteins in the RSV virus preparation elicits an inflammatory response. This enhanced inflammatory response might be unrelated to RSV and its localization to central sites (peribronchiolar and perivascular).

Of interest is that the FI-Vero cell vaccine-immunized animals showed greater inflammation centrally and less inflammation peripherally, as compared to animals with primary infection. The FI-RSV vaccine-enhanced inflammation was significantly greater than that of the primary infection at peribronchiolar, perivascular and interstitial sites. In contrast, tertiary infection in this animal model showed a protective effect from the prior RSV exposure. With the tertiary infection there was less inflammation at interstitial and alveolar sites as compared to primary infection. The pattern of protection from pulmonary inflammation in animals with tertiary infection shows a protective effect that is greatest at peripheral sites. Therefore, it appears that RSV-independent mechanisms can cause perivascular and peribronchiolar inflammation. This conclusion would suggest that an effective RSV vaccine should provide the least possible non-specific central pulmonary inflammatory enhancement while creating the greatest possible immune-specific peripheral pulmonary protection.

**Augmented virus replication in FI-RSV-immunized animals**

The finding of increased RSV replication in FI-RSV vaccine-immunized animals after RSV infection may be fundamental to understanding enhanced disease. The 10-fold increment in virus production in the lungs of the immunized animals is accompanied by an increase in titre in BAL samples with time. Prior studies in the African green monkey model using the A2 strain of RSV have shown similar increases in RSV titres in both lung and BAL samples from animals immunized with FI-RSV vaccine (Kakuk et al., 1993). In those animals 5–10-fold higher virus titres were observed as compared to lungs of animals immunized with an adjuvant alone.

The augmented virus replication documented by virus titres in the lungs of monkeys with FI-RSV vaccine-enhanced disease is further corroborated by the in situ hybridization results. In situ hybridization shows that the increased virus replication occurs at peribronchiolar and perivascular sites where immunostaining identified some of the inflammatory cells to be macrophages. In humans, macrophages have been shown to support virus replication (Becker et al., 1992; Midulla et al., 1989; Panuska et al., 1992) while other inflammatory cells such as lymphocytes do not seem to support a productive infection by RSV (Domurat et al., 1985; O’Donnell et al., 1998; Panuska et al., 1992). In the bonnet monkey the concentration of RSV message is greatest at perivascular and peribronchiolar sites. Comparable sections show that macrophages are the principal population in these sites of increased RSV replication.

The increase in RSV replication in perivascular sites of the FI-Vero cell vaccine group compared with the primary infection group was detected only by mRNA and not by virus culture. This is probably because quantitative results on hybridization for RSV mRNA were possible only in perivascular sites, and it is in these sites that the FI-Vero cell vaccine group animals develop an inflammatory response when challenged by infectious virus.

Previous studies in mice immunized with the FI-RSV vaccine, with the F or G protein of RSV or with vaccinia virus vectors expressing the F or G protein showed no evidence for increased virus replication after challenge with RSV, despite the occurrence of enhanced disease in the lungs (Graham et al., 1995; Hussell et al., 1997; Waris et al., 1996). Thus, the findings in the mouse model of FI-RSV disease contrast with those in primates. Mouse macrophages do not have a productive RSV infection in vivo (Franke-Ullmann et al., 1995). If this is true of macrophages in vivo, then FI-RSV vaccine-immunized mice might not show increased virus replication. The mechanisms for enhanced disease in mice and monkeys appear to be different. While there is concordance between the African green and bonnet monkey models, there is discordance between the primate and mouse model in the extent of virus replication following use of the FI-RSV vaccine. The concordance between enhanced virus replication in two separate non-human primate models of RSV disease emphasizes the importance of using primates rather than mice to evaluate vaccines and immunomodulators related to RSV disease.

**Immune response following immunization with FI-RSV vaccine**

Study of bonnet monkeys with tertiary infection shows that prior RSV respiratory exposure appears to stimulate very good antibody and lymphocyte proliferative responses, with no virus recovered from the lungs. Also the virus titres in the BAL samples remained low, with minimal inflammation seen at perivascular and peribronchiolar sites and the least inflammation at peripheral sites. In animals immunized with the FI-RSV high dose, there were very low amounts of antibody and low levels of proliferative response, with high levels of virus in the lungs and a temporal increase in virus titres in the BAL.
samples. There was also increased inflammation in all four intrapulmonary sites examined. In animals with low dose FI-RSV, there were moderate amounts of antibody and lymphocyte proliferative response, accompanied by lower virus titres in the lungs. The RSV titre in BAL of low dose FI-RSV animals remained low and inflammation did not extend to the alveoli. The trend in reduction of pathology to peribronchial and perivascular areas in animals with tertiary infection and animals immunized with low dose FI-RSV coupled with the absence of a temporal increase in virus titres in the BAL samples show that there is limited spread of virus from the initial central sites of replication to more peripheral sites. The blocking of virus spread peripherally may be attributed to the presence of anti-RSV antibody. If correct, this hypothesis emphasizes the importance of antibody production in any RSV-specific immunization protocol.

The bonnet monkey model did not reproduce the inverse relationship between susceptibility to enhanced disease and vaccine dose previously shown in mice (Fischer et al., 1997) and cotton rats (Murphy et al., 1990). In bonnet monkeys, it appears that antibody levels and T cell proliferation were greater at the low dose. Antibody and lymphocyte responses were lower in animals immunized with a high dose of FI-RSV vaccine, perhaps due to the induction of regulatory T cells or to some other suppressive mechanism. Our observation of low levels of functional antibody in FI-RSV-vaccinated animals mimics the response to the vaccine in human infants. Following two doses of FI-RSV vaccine neutralizing antibodies were found only in 17% of infants (Kim et al., 1969).

Enhanced RSV disease in mice has been attributed to alterations in the ratio of γ-IFN/IL-4-producing (Th1/Th2) cells (Fischer et al., 1997). In the FI-RSV vaccine–primed animals there is an increase in CD4 cells predominantly producing IL-4 (Graham et al., 1993; Srikiatkhachorn & Braciale, 1997a, b). The results with mice have lead to the speculation that enhanced pathology following FI-RSV vaccine in naive infants might also result from a change in cytokine balance (Chanock et al., 1992). The results from the bonnet monkey model suggest an alternative explanation. The enhanced pathology following FI-RSV vaccine immunization is in some way a consequence of increased replication of RSV within sites of inflammation. Increased virus load and infection of macrophages might augment the inflammatory response.

Antibody-mediated endocytosis is a likely mechanism by which RSV could enter macrophages. It is known that the IgG1 subclass of antibody binds to Fc receptors on macrophages (Van de Winkel & Capel, 1993) and therefore production of this subclass of antibody may be responsible for any augmented replication of RSV that might occur in macrophages of FI-RSV-immunized animals. The absence of augmented replication of RSV in mice, even when IgG1 predominates in the antibody response (as elegantly shown by Bembridge et al., 2001), argues for monkey studies in the evaluation of experimental RSV vaccines.

Evaluation of RSV vaccines in children could be dangerous. Our results show that the bonnet monkey model offers a reproducible alternative that mimics human disease but permits analysis of variables including immunogen route and attenuation, virus replication and immune response.

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In situ cells by cytokine mRNA expression patterns in lungs of mice challenged with formalin-inactivated virus or purified RSV F and G glycoprotein subgroups A and B: sequence conservation and divergence within RSV


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