Enhanced lung disease and Th2 response following human metapneumovirus infection in mice immunized with the inactivated virus

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Human metapneumovirus (hMPV) is a paramyxovirus that causes acute respiratory-tract infections in humans. The histopathological and immunological responses to hMPV infection in BALB/c mice immunized with inactivated hMPV were characterized. Animals were immunized intraperitoneally with PBS, supernatant from non-infected LLC-MK2 cells and from heat-inactivated influenza A- or hMPV-infected cells, all in incomplete Freund’s adjuvant, or with heat-inactivated hMPV without adjuvant, and then infected intranasally with 10^8 TCID50 virus. Following infection, lung samples and bronchoalveolar lavages were collected for determination of viral titre and cytokine levels and for histopathological studies. On day 1, 26% of mice immunized with inactivated hMPV and adjuvant died, compared with none in the other groups. There was more significant lung inflammation associated with eosinophilic infiltration, as well as increased levels of interleukin-4 (IL-4) and IL-5, in the bronchoalveolar lavages of mice immunized with hMPV alone or with the adjuvant. Mice from the last two groups had a 4–5 log10 decrease in their pulmonary viral titres compared with controls. Our data demonstrate the risks associated with immunization using inactivated hMPV in this animal model and that this aberrant response should be considered in the development of hMPV vaccines.

INTRODUCTION

Human metapneumovirus (hMPV) is a newly described member of the family Parainyoaviridae assigned to the genus Metapneumovirus in the subfamily Pneumovirinae (van den Hoogen et al., 2001). Since its discovery in 2001, hMPV has been identified in many countries from all continents, indicating its worldwide distribution (reviewed by Hamelin et al., 2004). hMPV is associated with acute respiratory-tract infections (ARTI) in all age groups, with more severe diseases such as bronchiolitis/bronchitis and pneumonia occurring in young children, elderly individuals and immunocompromised hosts (Boivin et al., 2003; Falsey et al., 2003; Larcher et al., 2005). Using RT-PCR methods, hMPV has been found in approximately 5% of children with upper respiratory-tract infections (URTI) (Williams et al., 2006), in 5–12% of those hospitalized for ARTI (Boivin et al., 2003; Williams et al., 2004) and also in persons with acute wheezing and asthma exacerbations (Jartti et al., 2002; Schildgen et al., 2006; Williams et al., 2005).

The hMPV genome consists of a single negative strand of RNA of approximately 13 kb, containing eight genes that are presumed to encode nine different proteins (Biacchesi et al., 2003; van den Hoogen et al., 2002). hMPV isolates can be separated into two major groups (A and B) and at least four subgroups (Boivin et al., 2004; Peret et al., 2002). Nucleotide and amino acid sequence identities between representative members of the two hMPV groups are 80 and 90%, respectively (Biacchesi et al., 2003). Of the three hMPV surface glycoproteins [fusion (F), attachment (G) and small hydrophobic (SH) proteins], the F protein is probably the major antigen that induces a protective immune response in hamsters and African green monkeys (Skiodopoulos et al., 2006; Tang et al., 2005).

Human respiratory syncytial virus (hRSV) is the most important respiratory pathogen in young children. This virus belongs to the same subfamily (Pneumovirinae) as hMPV, but to a different genus (Pneumovirus). In the 1960s, clinical trials of formalin-inactivated whole hRSV (FI-hRSV) vaccines adjuvanted with alum and administered intramuscularly revealed enhanced pulmonary disease in vaccinated infants upon subsequent hRSV infection (Kapikian et al., 1969; Kim et al., 1969). Enhanced pulmonary or atypical diseases have also been reported
with formalin-inactivated vaccines for other paramyxoviruses, such as measles virus (MV) (Fulginiti et al., 1967) and human parainfluenza virus type 3 (hPIV-3) (Ottolini et al., 2000), but not in the case of orthomyxoviruses (Edwards et al., 1994). These unexpected findings considerably slowed the development of vaccine programmes for hRSV for more than three decades (Englund, 2005). Experimental mouse models of immunization, including a control group corresponding to mice previously immunized with whole hRSV in incomplete Freund's adjuvant (ICFA) followed by viral infection, also resulted in increased eosinophil counts in bronchoalveolar lavages (BAL) (Openshaw et al., 1992). Recently, Yim et al. (2007) also observed a similar aberrant immune response in cotton rats previously infected with formalin-inactivated hMPV, indicating that this novel paramyxovirus seems to behave like other members of this family.

In a preliminary experiment on immunization using peptides combined with ICFA, we unexpectedly observed that control BALB/c mice previously immunized with heat-inactivated hMPV in ICFA developed a lethal infection, instead of being protected, following intranasal infection. This phenomenon was observed not only with ICFA, but also with Ribi adjuvant. In this study, we sought to characterize lung histopathological and immunological responses to hMPV infection in BALB/c mice previously immunized with whole, inactivated hMPV in ICFA.

**METHODS**

**Cell line and virus.** LLC-MK2 cells were maintained in minimum essential medium (MEM; Gibco–BRL) supplemented with 10% fetal bovine serum (FBS). HMPV C-85473 is a clinical strain (group A, as are CAN97-83 and NL/00-1) passaged seven times in LLC-MK2 cells using Opti-MEM medium (Gibco) supplemented with 0.0002% trypsin (Sigma) and 0.2% gentamicin (Gibco) (hMPV infection medium). A clinical influenza type A strain was also grown in LLC-MK2 cells. High virus titres for influenza and hMPV were obtained by infecting 20 flasks (75 cm²) of LLC-MK2 cells. High virus titres for influenza and hMPV were obtained by infecting 20 flasks (75 cm²) of LLC-MK2 cells until complete cytopathic effects were observed. Infected monolayers and supernatants were recovered with a cell scraper, sonicated and concentrated on Centricon columns (Fisher). The preparation was centrifuged on Centricon columns (Fisher). The preparation was centrifuged at 10 000 g for 10 min at 4°C, then cell pellets were resuspended in PBS + 10% PBS for leukocyte quantification using a haemocytometer. Specific cell populations were distinguished by their morphology, using haematoxylin- and eosin-stained cytospin preparations. The relative cell counts were calculated from a total of 2.5 × 10³ cells.

**Experimental protocol.** Groups of 42 4–6-week-old BALB/c mice (Charles River Laboratories) were immunized intraperitoneally every other week with 5 × 10⁶ TCID₅₀ heat-inactivated hMPV strain C-85473 with ICFA (hMPV–ICFA) or without ICFA (hMPV), for a total of three immunizations. As controls, similar groups of mice were immunized at the same time points with PBS (PBS–ICFA), non-infected LLC-MK2 cells (LLC-MK2–ICFA) or heat-inactivated influenza A virus (Flu–ICFA). Two weeks after the last immunization, blood samples were collected for determination of neutralizing-antibody titres and all animals were infected intranasally with 1 × 10⁶ TCID₅₀ hMPV strain C-85473 as described previously (Hamelin et al., 2005). At serial times post-infection (days 1, 2 and 3), BAL were performed in six mice per group to evaluate cytokine levels and leukocyte infiltration, and lung samples from another six mice per group were collected for histopathological studies. On day 5 post-infection, lungs were collected from six mice per group for determination of viral titres in cell culture.

**Virus titres in lungs.** On day 5 post-infection, which is the time of maximal viral replication in lungs of mice (Hamelin et al., 2005), animals were euthanized and their lungs were removed and frozen quickly in liquid nitrogen. Subsequently, lungs were weighed and homogenized in hMPV infection medium, and viral titres were determined by performing 10-fold serial dilutions of lung homogenates in 24-well plates containing LLC-MK2 cells. Before infection, cells were washed twice with PBS. Infected plates were incubated at 37°C with 5% CO₂ and replenished with 1 μl fresh trypsin (0.0002%) every other day. Virus titres were reported as log₅₀ TCID₅₀ (g lung)⁻¹. The lower limit of detection of this assay is 10⁶ TCID₅₀ (g lung)⁻¹. TCID₅₀ values were calculated by the method of Reed & Muench (1938).

**Neutralizing-antibody assay.** HMPV neutralizing-antibody titres were determined by an end-point dilution assay (Skiadopoulos et al., 2006). Briefly, blood samples were centrifuged (1200 r.p.m., 10 min), then sera were collected and incubated at 56°C for 30 min to inactivate non-specific inhibitors. Twofold dilutions of sera were performed in quadruplicate in hMPV infection medium, and then mixed with an equal volume of infection medium containing 500 TCID₅₀ hMPV strain C-85473. Those virus–antibody mixtures were incubated at 37°C for 2 h and then transferred into 24-well plates containing LLC-MK2 cells. Following incubation for 5 h, the mixtures were removed and 50 μl fresh infection medium was added to each well. Plates were incubated at 37°C and, 3 days later, another 500 μl infection medium was added with fresh trypsin. Neutralization titres were defined as the highest dilution of antibody at which culture wells were negative for infection. hMPV neutralizing-antibody titres were reported as mean reciprocal log₂ titres ± SD. The lower limit of detection of this assay is 3 log₂.

**Types of inflammatory cell in BAL.** Cell recruitment in BAL samples was determined in six mice per group on days 1, 2 and 3 post-infection. A catheter was first placed in the trachea then 1.0 ml injections of PBS were performed three times. BAL samples were centrifuged at 1800 g for 10 min at 4°C, then cell pellets were resuspended in PBS + 10% FBS for leukocyte quantification using a haemocytometer. Specific cell populations were distinguished by their morphology, using haematoxylin- and eosin-stained cytospin preparations. The relative cell counts were calculated from a total of 2.5 × 10³ cells.

**Pulmonary cytokine levels.** BAL samples were centrifuged at 13 800 g for 10 min at 4°C, then 50 μl supernatant was used for cytokine quantification. Levels of interleukin-2 (IL-2), IL-4, IL-5, IL-10, IL-12, IL-13 and gamma interferon (IFN-γ) were determined by the use of multiplex mouse Th1/Th2 6-plex and IL-13 antibody bead kits (Biosource) according to the manufacturer's instructions. Experiments were performed in a 96-well filter plate and results were analysed with the Luminex system (Qiagen).

**Pulmonary histopathology.** Lungs were removed and fixed with 10% buffered formalin. Fixed lungs were embedded in paraffin, sectioned in 4 μm slices and stained with haematoxylin and eosin. The histopathological scores (HPS) were determined by two independent pathologists with experience in pulmonary pathology, who were unaware of the infection status of the animals. A semiquantitative scale was used to score bronchial/endobronchial, peribronchial, perivascular, interstitial, pleural and intra-alveolar inflammation. Capillary vascular congestion and pulmonary oedema were also evaluated by using a semiquantitative scale, and the inflammatory cellular infiltrate was characterized to determine
whether the inflammation was acute (neutrophilic) or chronic (lymphohistiocytic) (Table 1).

**Statistical analysis.** All data, with the exception of histopathological scores, are expressed as mean ± SD. The Mann–Whitney U test was used to compare all numerical values between groups of mice at each time point, whereas the χ² test was used to compare mortality rates between groups.

**RESULTS**

**Clinical manifestations of enhanced pulmonary disease in BALB/c mice**

hMPV-infected mice were observed on a daily basis for up to 5 days post-infection to assess clinical signs in groups of mice that were immunized with heat-inactivated hMPV alone, hMPV–ICFA, PBS–ICFA, LLC-MK2–ICFA and Flu–ICFA. A total of 11 (26%) hMPV–ICFA mice died rapidly following hMPV infection (nine within 24 h, one on day 2 and one on day 3), whereas no deaths occurred in mice from the other four groups (P<0.01). On day 1 post-infection, all hMPV–ICFA mice had a pronounced decrease in their physical activity, a tendency to huddle and very ruffled fur compared with PBS–ICFA mice, for which only lightly ruffled fur was observed. LLC-MK2–ICFA, Flu–ICFA and hMPV mice also presented these clinical signs, which were less marked than those of hMPV–ICFA mice, but more pronounced than those of PBS–ICFA mice. On the other hand, surviving hMPV–ICFA mice did not present any signs of illness on day 5 post-infection. This was in sharp contrast to PBS–ICFA mice, which had maximal symptoms at this time point, as reported previously during the course of natural hMPV infection (Hamelin et al., 2005).

**Lung viral titres**

Five days after intranasal infection with 10⁶ TCID₅₀ hMPV, the mean viral titres in lungs of surviving mice that had received heat-inactivated hMPV–ICFA, hMPV, PBS–ICFA, LLC-MK2–ICFA and Flu–ICFA were 2.96 × 10² ± 1.88 × 10², 4.35 × 10⁵ ± 2.2 × 10⁵, 1.62 × 10⁷ ± 1.84 × 10⁷, 9.17 × 10⁶ ± 2.45 × 10⁶ and 8.7 × 10⁶ ± 3.1 × 10⁶ TCID₅₀ (g lung)⁻¹, respectively (P<0.05) (Table 2).

**Neutralizing-antibody titres**

After three immunizations containing hMPV with and without ICFA, mice had developed significant hMPV neutralizing antibodies, with a mean reciprocal titre of 7.0 log₂ ± 0.8 log₂ and 5.5 log₂ ± 0.4 log₂, respectively (Table 2). In contrast, mice immunized with non-infected LLC-MK2 cells, influenza virus or PBS in ICFA did not have detectable neutralizing antibodies against hMPV (titre <3 log₂) (P<0.05).

**Cellular infiltration in BAL samples**

To characterize the inflammatory response following hMPV infection, the recruitment of inflammatory cells in BAL specimens from mice was monitored during the period when mortality was noted (days 1–3) (Fig. 1). A significant infiltration of eosinophils was observed over time in BAL of hMPV- and hMPV–ICFA-immunized mice, with up to 20 ± 6.26 and 18.33 ± 2.62 % of eosinophils, respectively, on day 3 compared with 6.25 ± 3.34 % in LLC-MK2–ICFA mice (P<0.05), 4 ± 2.28 % in Flu–ICFA mice (P<0.05) and 1.00 ± 0.63 % in PBS–ICFA mice (P<0.05). In addition, the eosinophils were observed to be hyperlobulated. Monocyte/macrophage cell counts were also increased significantly in LLC-MK2–ICFA, Flu–ICFA, hMPV–ICFA and hMPV mice on day 1 compared with PBS–ICFA mice, whereas neutrophil counts were reduced significantly at that time in the former groups. Lymphocyte counts increased slowly from day 1 to day 3 in all groups, without any significant differences.

**Levels of cytokines in BAL samples**

Levels of various cytokines were assessed to characterize the immunological profile corresponding to enhanced

**Table 1. Semiquantitative scoring scale of histological parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0; normal/absent</th>
<th>1; mild</th>
<th>2; moderate</th>
<th>3; marked</th>
</tr>
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<tbody>
<tr>
<td>Inflammation intensity*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bronchial/endo bronchial</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peribronchial</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Perivascular</td>
<td></td>
<td></td>
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<tr>
<td>Interstitial</td>
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<tr>
<td>Pleural</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Intra-alveolar</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type of inflammatory cellular infiltrate</td>
<td>NA; not applicable</td>
<td>A; acute (neutrophilic)</td>
<td>C; chronic (lymphohistiocytic)</td>
<td>AC; acute and chronic</td>
</tr>
<tr>
<td>Capillary vascular congestion*</td>
<td>0; absent</td>
<td>1; mild</td>
<td>2; moderate</td>
<td>3; marked</td>
</tr>
<tr>
<td>Pulmonary oedema*</td>
<td>0; absent</td>
<td>1; mild</td>
<td>2; moderate</td>
<td>3; marked</td>
</tr>
</tbody>
</table>

*Fractions of points are allowed for very mild (0.5), mild to moderate (1.5) and moderate to marked (2.5).
pulmonary disease (Fig. 2). IL-2 was increased significantly in hMPV–ICFA mice on day 3 post-infection compared with other groups of mice. Levels of the Th1 cytokine IFN-\(\gamma\) were significantly lower on day 3 in hMPV–ICFA mice compared with other groups of mice, as were levels of IL-12 in hMPV–ICFA and hMPV mice compared with controls. In contrast, levels of the Th2 cytokines IL-4 and IL-5 were significantly higher in hMPV–ICFA and hMPV mice than in control mice, with maximal expression on day 1 post-infection for IL-4 and on day 2 for IL-5. Levels of IL-10 and IL-13 were only significantly increased in hMPV–ICFA mice compared with other groups on day 1 and days 1 and 2, respectively.

**Histopathological changes in lungs**

Pulmonary inflammation was assessed by using the scoring scale described in Table 1. Histopathological scores were

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Viral lung titre* (mean ± SD TCID(_{50}) g(^{-1}))</th>
<th>Neutralizing-antibody titre† (mean ± SD reciprocal log(_2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMPV–ICFA</td>
<td>(2.96 \times 10^2 \pm 1.88 \times 10^2)‡</td>
<td>7.0 ± 0.8‡</td>
</tr>
<tr>
<td>hMPV without adjuvant</td>
<td>(4.35 \times 10^2 \pm 2.20 \times 10^2)‡</td>
<td>5.5 ± 0.4‡</td>
</tr>
<tr>
<td>PBS–ICFA</td>
<td>(1.62 \times 10^2 \pm 1.84 \times 10^2)</td>
<td>&lt;3.0</td>
</tr>
<tr>
<td>LLC-MK2–ICFA</td>
<td>(9.17 \times 10^6 \pm 2.45 \times 10^6)</td>
<td>&lt;3.0</td>
</tr>
<tr>
<td>Flu–ICFA</td>
<td>(8.7 \times 10^6 \pm 3.1 \times 10^6)</td>
<td>&lt;3.0</td>
</tr>
</tbody>
</table>

* Determined on day 5 post-infection. Limit of detection, \(10^2\) TCID\(_{50}\) g\(^{-1}\).
† Analyzed before infection and after three immunizations at 2 week intervals. Limit of detection, 3 log\(_2\).
‡ \(P<0.05\) compared with PBS–ICFA, LLC-MK2–ICFA and Flu–ICFA adjuvant groups.

Fig. 1. Recruitment of inflammatory cells in bronchoalveolar lavages (BAL) of hMPV-infected mice previously immunized with IFA and PBS (PBS–ICFA; empty bars), supernatant from non-infected LLC-MK2 cells (LLC-MK2–ICFA; shaded bars), heat-inactivated influenza virus (Flu–ICFA; filled bars) or heat-inactivated hMPV with adjuvant (hMPV–ICFA; diagonally hatched bars) or without adjuvant (hMPV; horizontally hatched bars). Four to six mice per group were euthanized at different times post-infection (days 1, 2 and 3) and BAL fluids were collected. Total leukocyte populations were determined by using a haemocytometer, then 2.5\(\times\)10\(^5\) cells were spotted on a slide with a cytopsin. Specific cell populations were distinguished by their morphology using an haematoxylin and eosin stain preparation. Results are shown as means±SD. Statistically significant differences (\(P<0.05\)) were observed (*) between PBS–ICFA mice and the other groups of mice or (‡) between LLC-MK2–ICFA and Flu–ICFA and the other groups of mice (hMPV–ICFA and hMPV), based on the Mann–Whitney U test.
Fig. 2. Cytokine levels in bronchoalveolar lavages (BAL) of hMPV-infected mice previously immunized with ICFA and PBS (PBS–ICFA; empty bars), supernatant from non-infected LLC-MK2 cells (LLC-MK2–ICFA; shaded bars), heat-inactivated influenza virus (Flu–ICFA; filled bars), or heat-inactivated hMPV with adjuvant (hMPV–ICFA; diagonally hatched bars) or without adjuvant (hMPV; horizontally hatched bars). Four to six mice per group were euthanized at different times post-infection (days 1, 2 and 3) and BAL fluids were collected. Samples (50 µl) were used to quantify IL-2, IL-4, IL-5, IL-10, IL-12, IL-13 and IFN-γ by using the Luminex system (Qiagen). Th1 (a) and Th2 (b) cytokine profiles were compared. Statistically significant differences (\( P < 0.05 \)) were observed (*) between PBS–ICFA mice and the other groups of mice or (#) between LLC-MK2–ICFA and Flu–ICFA and the other groups of mice (hMPV–ICFA and hMPV), based on the Mann–Whitney U test.
determined on days 1, 2 and 3 post-infection to characterize the acute inflammatory reaction following intranasal infection. At 24 h post-infection, hMPV–ICFA mice had more pronounced pulmonary inflammation, characterized by increased peribronchial, perivascular, interstitial and intra-alveolar inflammation compared with other groups of mice [Figs 3(a) and 4]. Following hMPV infection, significant pulmonary oedema and vascular congestion were also observed on days 1–3 post-infection in hMPV–ICFA mice (Fig. 3b). These pathological manifestations were also noted in LLC-MK2–ICFA, Flu–ICFA and hMPV mice but, in contrast to hMPV–ICFA mice, they increased from day 1 to day 3, whereas pulmonary oedema was scarcely observed in PBS–ICFA mice (Figs 3 and 4). The inflammatory cellular infiltrates observed in hMPV–ICFA mice were consistent with an acute inflammation (mainly neutrophilic), whereas they were mainly lymphohistiocytic in PBS–ICFA mice (not shown). LLC-MK2–ICFA, Flu–ICFA and hMPV mice presented both types of inflammation.

**DISCUSSION**

We characterized the histopathological and immunological responses to hMPV infection in BALB/c mice previously immunized with whole, heat-inactivated virus in ICFA (hMPV–ICFA). Enhanced pulmonary disease was observed in these mice following intranasal infection with 10⁸ TCID₅₀ virus, resulting in a 26% mortality rate. On day 1 post-infection, all hMPV–ICFA mice presented obvious clinical signs, such as a decrease in physical activity, a tendency to huddle and very ruffled fur compared with control mice that had been immunized with PBS (PBS–ICFA) or supernatant from non-infected LLC-MK2 cells (LLC-MK2–ICFA), or infected with influenza A virus (Flu–ICFA) or with hMPV without adjuvant (hMPV). The hMPV–ICFA and hMPV mice also had more pronounced pulmonary inflammation shortly after infection, with a biased Th2 cytokine immune response. Overall, our data suggest that immunization with inactivated hMPV induced a severe pulmonary disease following intranasal hMPV infection and that such a response can be potentiated by our adjuvant (ICFA).

Enhanced pulmonary and atypical diseases have been observed following paramyxovirus infections in different animal models after immunization with formalin-inactivated vaccines containing whole viruses (De Swart et al., 2002; Ottolini et al., 2000; Waris et al., 1996). In a preliminary experiment on immunization, we unexpectedly observed similarly enhanced disease following hMPV infection in mice immunized with ICFA and PBS (PBS–ICFA), supernatant from non-infected LLC-MK2 cells (LLC-MK2–ICFA), heat-inactivated influenza virus (Flu–ICFA) or heat-inactivated hMPV with (hMPV–ICFA) or without adjuvant (hMPV). The degree of lung inflammation (mean histopathological score) was evaluated for peribronchial (empty bars), perivascular (shaded bars), interstitial (filled bars), intra-alveolar (diagonally hatched bars) and pleural (horizontally hatched bars) inflammation (a), as well as for vascular congestion (empty bars) and pulmonary oedema (filled bars) (b). Histopathological scores were determined as described in Table 1.

![Histopathological scores](image-url)
infection in a control group of mice immunized with whole, heat-inactivated virus in ICFA. Although ICFA is not used for immunization in humans, we sought to characterize the histopathological and immunological features of this enhanced pulmonary disease to illustrate the adverse effects that some adjuvants might have. We have also observed such aberrant immune responses following the use of other vaccine preparations, including Ribi. No deaths occurred in Ribi-immunized mice following viral infection, but severe clinical signs and a significant decrease in activity were noted compared with non-immunized mice. Thus, we consider it of importance to report such enhanced disease in the context of potential development of inactivated hMPV vaccines. These results might not be related directly to the enhanced disease observed previously with inactivated hRSV vaccine and alum, but certainly demonstrate that adjuvants can modulate hMPV response in an aberrant way. Also, Yim et al. (2007) recently reported that infection following immunization with formalin-inactivated hMPV induces an aberrant immune response in cotton rats, characterized by a significant Th2 response and pronounced pulmonary inflammation, similar to the phenomenon that we observed in mice.

Mice immunized with hMPV alone (hMPV) or with the adjuvant (hMPV–ICFA) had a 4 or 5 log10 decrease in pulmonary viral titres on day 5 post-infection, respectively, and higher neutralizing-antibody titres (5.5 log2 ± 0.4 log2 and 7 log2 ± 0.8 log2, respectively) than control mice (Table 2). Thus, most immunized mice seemed to be protected against viral infection, but presented a more pronounced and early pulmonary inflammation than controls (Figs 3 and 4). Such an inverse relationship between decreased viral titres and enhanced pulmonary disease has been observed in other animal models (Prince et al., 2001) and may indicate an allergic sensitization, rather than the viral pathogenesis normally observed during primary infection. The immune response to hMPV infection has already been shown to lead to significant airway hyper-responsiveness (Hamelin et al., 2006) and a biased Th2 cytokine response (Alvarez & Tripp, 2005). The latter features are also reminiscent of allergic sensitization, and priming mice with immunizations using the whole virus could sensitize them to a further aberrant immune response following hMPV infection.

Enhanced pulmonary inflammation was most evident in hMPV–ICFA mice and consisted of marked interstitial, intra-alveolar and perivascular inflammation with significant pulmonary oedema on day 1 post-infection that decreased thereafter compared with other groups of mice. In contrast to hMPV–ICFA mice, LLC-MK2–ICFA, Flu–ICFA and PBS–ICFA mice presented increased pulmonary inflammation over time, which is reminiscent of the typical pathogenesis observed during primary infection, rather than allergic sensitization. hMPV immunization without the adjuvant induced an early (day 1) pulmonary inflammation at a level between that of hMPV–ICFA and controls, and the most important inflammatory changes occurred on day 3, which indicates that the inactivated virus itself might induce an aberrant response. In other experimental models of hRSV infection, including BALB/c

**Table 2.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1 post-infection</th>
<th>Day 2 post-infection</th>
<th>Day 3 post-infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS–ICFA–immunized mice</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
</tr>
<tr>
<td>LLC-MK2–ICFA–immunized mice</td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>Flu–ICFA–immunized mice</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
</tr>
<tr>
<td>hMPV–ICFA–immunized mice</td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
</tr>
<tr>
<td>hMPV–immunized mice</td>
<td><img src="image13" alt="Image" /></td>
<td><img src="image14" alt="Image" /></td>
<td><img src="image15" alt="Image" /></td>
</tr>
</tbody>
</table>

**Fig. 4.** Lung histopathology of hMPV-infected mice previously immunized with ICFA and PBS (PBS–ICFA), supernatant from non-infected LLC-MK2 cells (LLC-MK2–ICFA), heat-inactivated influenza virus (Flu–ICFA) or heat-inactivated hMPV with adjuvant (hMPV–ICFA) or without adjuvant (hMPV). Mice were euthanized on days 1, 2 and 3 post-infection and their lungs were removed and fixed with 10% formalin. Thin sections of paraffin-embedded lung tissues were cut and stained with haematoxylin and eosin. Representative sections (×10 enlargement) are shown for the different groups of mice on days 1–3 post-infection.
mice, cotton rats and cynomolgus macaques immunized with formalin-inactivated whole hRSV (FI-RSV), enhanced pulmonary inflammation, sometimes associated with mortality (cynomolgus macaques only), was also observed following viral infection (Connors et al., 1994; De Swart et al., 2002; Prince et al., 2001). However, pathological conditions occurred at later times post-infection in these hRSV studies (days 4, 4–5 and 7–12 post-infection in BALB/c mice, cotton rats and macaques, respectively) compared with our hMPV–ICFA findings and this might be due to intrinsic viral or host factors, different viral inocula or different vaccine preparations (Freund’s adjuvant versus formalin-inactivated virus). The clinical and histopathological results obtained with hMPV immunizations without adjuvant are different from those with hMPV–ICFA and are more related to the findings noted previously with inactivated hRSV vaccines.

Analysis of cytokine levels in BAL following viral infection of hMPV- and hMPV–ICFA-immunized mice revealed a significantly biased Th2 response compared with controls. Indeed, there were markedly increased levels of IL-4 and IL-5 as soon as day 1 post-infection (Fig. 2). Immunization with supernatant from non-infected LLC-MK2 cells and influenza A virus grown in this cell line also induced an increase of those cytokines following hMPV infection; however, such levels were significantly lower than those observed in hMPV- and hMPV–ICFA-immunized mice, indicating that the virus itself or the combination of hMPV antigens with the adjuvant induced a strong Th2 response. Notably, significant increases in IL-10 and IL-13 were only observed in hMPV–ICFA-immunized mice, which illustrates that the adjuvant potentiates the immune response caused by viral antigens. IL-5 is a haemato poetic factor responsible for growth and differentiation of eosinophils, and the increased production of this cytokine was also correlated with increased eosinophil counts in BAL of hMPV- and hMPV–ICFA-immunized mice (Fig. 1). The eosinophils observed under light microscopy had typical pink granular staining, but were hyperlobulated, which is often associated with eosinophilic infiltration, sometimes associated with bronchial asthma (Sparrevohn & Wulff, 1967). Interestingly, this phenomenon has also been observed after immunization with whole hRSV in ICFA (Openshaw et al., 1992) or with the FI-RSV vaccine in BALB/c mice (Waris et al., 1996) and cynomolgus macaques (De Swart et al., 2002).

The levels of the Th1 cytokine IL-12 were significantly lower in hMPV- and hMPV–ICFA-immunized mice than in control mice on days 2–3 post-infection (Fig. 2). In contrast to IL-12, IFN-γ and IL-2 levels were significantly different only in hMPV–ICFA-immunized mice compared with controls, indicating the effect of the adjuvant on hMPV response. The IFN-γ response to hMPV infection has been reported previously to be low compared with that to hRSV infection (Douville et al., 2006) and immunization with the virus in ICFA seemed to reduce this response in a more significant way. Immunization of BALB/c mice with PBS and ICFA did not seem to modify the immunological response seen during primary hMPV infection, as no detectable levels of IL-4 and IL-5 or similar increased production of IL-2 and IFN-γ were observed from days 1 to 3 post-infection in both PBS–ICFA-immunized and unimmunized mice (Guerrero-Plata et al., 2005; Hamelin et al., 2001).

Following immunization, hMPV and hMPV–ICFA mice became hypersensitive to hMPV infection. It has been reported that formalin-inactivated vaccines, such as non-viral proteins or FBS, present in immunization preparations can predispose rodents to hypersensitivity upon hRSV challenge with the same preparation (Piedra et al., 1993). Although cellular components could contribute to the enhanced disease phenomenon observed following viral infection in immunized animals, this is certainly not the only mechanism, as immunization with formalin-inactivated viruses from other viral families did not induce enhanced pathology following viral challenge (Edwards et al., 1994; Gruber et al., 1990), and children who died in the 1960s were not infected with the same viral stock used for immunization (Kapikian et al., 1969; Kim et al., 1969). Other groups have also reported that the presence of cellular proteins in FI-RSV vaccines was not responsible for enhanced inflammation (Connors et al., 1994; Waris et al., 1996) and that large amounts of cellular extracts were needed to produce a similar aberrant response (Vaux-Peretz et al., 1992). We cannot completely exclude the possibility that some of the immunopathological changes observed in our model were due to recall response to non-viral proteins. Indeed, we observed significant eosinophilic infiltration and a Th2-like cytokine response in BAL, as well as inflammation in lungs, of LLC-MK2–ICFA and Flu–ICFA mice compared with PBS–ICFA mice, although such changes were significantly less marked than those found in hMPV and hMPV–ICFA mice.

Previous experiments, aimed at understanding better the underlying mechanisms responsible for the enhanced pulmonary disease seen with the inactivated hRSV vaccine, have resulted in different hypotheses. Some groups have reported that priming with complete (Johnson & Graham, 1999; Openshaw et al., 1992) or partial (Tebby et al., 1998) sequences of glycoprotein G, as well as with purified glycoprotein F (Murphy et al., 1990), induced a Th2-type cytokine response with eosinophilic infiltration following subsequent hRSV infection. However, other investigators could not confirm such findings using G (Johnson et al., 2004), F (Hildreth et al., 1993) or even a chimeric FG (Kakuk et al., 1993) glycoprotein. Finally, it has been suggested that the antigenic preparation and the route of immunization could influence eosinophilic infiltration in the lungs following hRSV challenge (Bembridge et al., 1998) and that antigen delivery, not only protein composition, should be considered. Our results also indicate that the choice of adjuvant must be considered carefully, as it may induce an undesired response. Thus, a specific protein does not seem to be the only factor...
responsible for the enhanced pulmonary disease seen in most studies, and such pathological phenomena might well be multifactorial.

In conclusion, viral infection of mice previously immunized with heat-inactivated hMPV with or without ICFA results in enhanced pulmonary disease associated with an important eosinophilic infiltration in BAL and a Th2-cytokine response. This hypersensitive response is not a phenomenon unique to hMPV or to our mouse model, as it has also been observed upon viral challenge in humans and various other animals immunized with different paramyxovirus preparations (Fulginiti et al., 1967; Kapikian et al., 1969; Kim et al., 1969; Ottolini et al., 2000; Polack et al., 1999; Waris et al., 1996). These observations suggest that, if used, inactivated vaccines should be given with adjuvants that modulate the immune response to minimize the risk of enhanced pathological disease (Oumouna et al., 2005). The use of subunit, live-attenuated (Biacchesi et al., 2005) or recombinant (Skiadopoulos et al., 2006) viruses could be safer options to pursue in the development of hMPV vaccines. Further studies are still required for a better understanding of the mechanisms leading to aberrant immunological responses to paramyxovirus infections following immunization with inactivated vaccines.

**ACKNOWLEDGEMENTS**

This study was supported by research grants from the Canadian Institutes of Health Research (MOP-62789) and by ‘Le Fonds de la Recherche en Santé du Québec’ (FRSQ-Respiratory Health Network) to G. B. G. B. is a senior researcher scholar of the FRSQ and the holder of the Canadian Research Chair on Emerging Viruses and Antiviral Resistance. M.-E. H. has a PhD scholarship from the FRSQ.

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