Local immune response to respiratory syncytial virus infection is diminished in senescence-accelerated mice

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The effect of ageing on the local defence system against respiratory syncytial virus (RSV) infection was investigated using an aged mouse model of the senescence-accelerated mouse (SAM) strain P1. Following intranasal infection with RSV, SAM-P1 mice showed a marked loss in weight, with elevated virus growth in the lungs and prolonged virus shedding. The increased susceptibility to RSV infection was associated mainly with diminished cellular immunity by local virus-specific cytotoxic T lymphocytes and natural killer cells. The deficiency in cellular immune responses was due to a lack of clonal expansion of CD4⁺ and CD8⁺ T lymphocytes, together with an imbalance of T-helper type 1 (Th1)/Th2 cytokine production in the respiratory tract, including the lungs. Furthermore, the production of virus-specific local IgA antibody was restrained. Prolonged virus loading in the lungs of SAM-P1 mice caused a massive infiltration of CD16⁺/32⁺ inflammatory cells, which was one factor responsible for severe pneumonia. The adoptive transfer of immune-competent spleen cells achieved an appreciable protection for SAM-P1 mice against RSV challenge infection. These results suggested that age-related immune dysfunction, especially defects in cellular immune responses, accounts for the increased morbidity and mortality in RSV infection of the elderly.

INTRODUCTION

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract diseases in infants and young children, but also causes substantial morbidity and mortality in elderly people (Falsey et al., 1995, 2005; Dowell et al., 1996). RSV infection is associated with 10% of elderly people hospitalized for acute cardiopulmonary condition and with 13% hospitalized for influenza (Walsh et al., 1999). The reasons for more-severe clinical disease with RSV infection in the elderly are not well understood. Age-related immune dysregulation (Miller, 1996; Boukhvalova et al., 2007) seems likely to be the most important factor in association with infectious diseases. It has been shown that elderly people exhibit a significant deficiency in the influenza virus-specific CD8⁺ T-cell response when compared with young adults (Mbawuike et al., 1993). Experimentally, aged mice are more susceptible to influenza virus as well as RSV infection, exhibiting a deficiency in virus-specific cytotoxic T lymphocyte (CTL) responses with small amounts of gamma-interferon (IFN-γ) but large amounts of interleukin (IL)-4 production (Mbawuike et al., 1996; Zhang et al., 2002). However, local immune responses to RSV infection in the elderly have not been well investigated. To examine the age-associated immune alteration and its influence on defence mechanisms against RSV infection, we adopted a newly developed senescence-accelerated mouse (SAM) system. The senescence-prone strain of SAM mice, SAM-P1, shows various signs of rapid ageing, such as a shortened lifespan of about half that of ordinary control mice, wrinkled skin and age-dependent geriatric disorders, including senile amyloidosis of the joints, hyperinflation of the lungs, hearing impairment and immune deficiency (Abe et al., 1994; Haruna et al., 1995). SAM-P1 mice show an age-related decline in immune responses, particularly cellular immune responses, as early as 2 months old (Toichi et al., 1997; Dong et al., 2000). These characteristics of the SAM-P1 mice are useful for animal model experiments involving virus infection among geriatric patients.

In the present study, we infected SAM-P1 mice intranasally with RSV and investigated the host defence system, particularly focusing on the local immune response in the respiratory tract, as RSV infection is restricted to surface infection of the airway mucosal membrane cells but not viraemia. Age-matched parental senescence-regular SAM-R1 mice were used as a control.
METHODS

Virus. Human RSV type A2 was kindly supplied by Dr K. Hashimoto, Fukushima Medical University School of Medicine, Fukushima, Japan. The virus was propagated in monolayers of HEp-2 cells grown in Eagle’s minimum essential medium (Nissui Pharmacia) supplemented with 2 % heat-inactivated fetal calf serum (Greiner). At the time point of maximum cytopathic effect, cells were harvested and disrupted by sonication in the same culture medium. The suspension was clarified by centrifugation at 2000 g for 20 min at 4 °C and the resulting supernatant was layered on top of a sucrose gradient (30 % sucrose in 50 mM Tris-buffered normal saline solution containing 1 mM EDTA, pH 7.5) and further centrifuged at 100 000 g for 2 h at 10 °C. The pellet was resuspended in 10 mM PBS containing 15 % sucrose and stored in aliquots at −80 °C. Virus titre was determined and expressed as a 50 % tissue culture infectious dose (TCID₅₀), calculated using the method of Reed & Muench (1938).

To inactivate the virus, an aliquot of the virus suspension was irradiated with UV light for 15 min on ice (Reuman et al., 1990). After irradiation, no infectivity could be detected.

Mice. The senescence-prone SAM-P1 strain (H₂-k), with a genetic background of A/KRJ mice, and its parental senescence-regular SAM-R1 strain were obtained from the Institute for Frontier Medical Science (Kyoto University, Japan). Mice had fresh water and autoclaved food and were kept at 23 °C under conventional conditions throughout all experiments. Three-month-old SAM-P1 mice and age-matched SAM-R1 mice were used in this study. Mice were mildly anaesthetized by intraperitoneal administration of pentobarbital sodium (0.025 mg g body weight⁻¹) and inoculated in the right nostril with 20 μl PBS containing 2 × 10⁶ TCID₅₀ RSV per mouse. At intervals, lung tissue was removed aseptically. Lung homogenates were prepared in a mortar using sterile sea sand and collected in 2 ml sterile PBS. After centrifugation at 1250 g for 10 min, supernatants were frozen at −80 °C until the virus titre was assayed. To avoid laboratory contamination, all virus-infected mice were housed in negatively pressurized isolators equipped with a ventilation system through a high-efficiency particulate air filter (AH model; Nihon-Ika). This work was approved by the Institutional Animal Care and Use Committee of Fukui University School of Medicine, Japan.

Preparation of single-cell suspensions from the lung parenchyma. Mice were anaesthetized and the lung was flushed aseptically. Lung homogenates were disrupted by sonication in the same culture medium. The suspension was clarified by centrifugation at 2000 g for 20 min at 4 °C and the resulting supernatant was layered on top of a sucrose gradient (30 % sucrose in 50 mM Tris-buffered normal saline solution containing 1 mM EDTA, pH 7.5) and further centrifuged at 100 000 g for 2 h at 10 °C. The pellet was resuspended in 10 mM PBS containing 15 % sucrose and stored in aliquots at −80 °C. Virus titre was determined and expressed as a 50 % tissue culture infectious dose (TCID₅₀), calculated using the method of Reed & Muench (1938).

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Preparation of single-cell suspensions from the lung parenchyma. Mice were anaesthetized and the lung was flushed in situ with 20 ml sterile PBS via cannulation of the heart to remove the intravascular blood pool. Minced lung tissues were incubated at 37 °C for 60 min on a rocker with 200 μg collagenase D ml⁻¹ and 40 μg DNase ml⁻¹ (both from Roche Molecular Biochemicals) as described previously (Liu et al., 2004). Subsequently, the enzyme-digested lung tissue was passed through a stainless steel mesh. Single-cell suspensions were collected by density-gradient centrifugation with lymphocyte separation solution (Antibody Institute).

Identification of lung parenchymal cells. Each separate aliquot of lung parenchymal cells was incubated on ice for 20 min with the following monoclonal antibodies (mAbs); phycoerythrin-labelled mAb for CD4 and fluorescein isothiocyanate-labelled mAb for CD8, CD16/32 or CD19 (Caltag Laboratory). The fluorescence intensity of cell samples was assayed on a fluorescence-activated cell sorter (EPICS XL; Beckman Coulter), acquiring 10 000 events per sample. Data were analysed using the computer program SYSTEM 2, version 1.0.

Assay of cytokine production. Single-cell suspensions (4 × 10⁵ cells in 200 μl per well) were prepared from the lung parenchyma of SAM mice and cultured for 48 h in the presence of UV-inactivated RSV antigens (equivalent to the original of 2 × 10⁶ TCID₅₀ per well). The supernatants were then harvested and assayed for IFN-γ and IL-4 titres using a mouse cytokine detection ELISA kit (BioSource International) in accordance with the manufacturer’s instructions.

Assay of CTL activity. A cytotoxicity assay was performed according to a protocol described previously (Liu et al., 2001). Lung parenchymal cells were collected from infected mice. Mouse l.29 (H₂-K¹) cells infected with RSV at an input m.o.i. of 1 TCID₅₀ were used as target cells. Lymphocytes and target cells were mixed and incubated at 37 °C in a 5 % CO₂ atmosphere for 4 h. Specific lysis of target cells was determined by a lactate dehydrogenase-release assay (Decker & Lohmann-Matthes, 1988) using a cytotoxic detection kit (Roche Applied Science). Data were expressed as the percentage of specific release using the following formula: cytotoxicity (%) = 100 × [target with effector–effector spontaneous]–target spontaneous/[target maximum–target spontaneous].

Assay of NK cell activity. Lung parenchymal cells were collected 3 days after infection and co-cultured with NK-sensitive Yac-1 target cells at 37 °C for 4 h. Specific lysis of target cells was determined by a lactate dehydrogenase-release assay as described above.

Antibody assay. Virus-specific immunoglobulins (Igs) were measured using an ELISA Ig Quantitative kit (Bethyl Laboratories). Briefly, microtitre plates were coated with 10 μg purified RSV proteins overnight at 4 °C. After blocking with 1 % BSA for 30 min, bronchoalveolar lavage (BAL) fluids were added to the well and incubated for 1 h. Bound antibodies were reacted with goat horse-radish peroxidase-labelled anti-mouse IgG1, IgG2a or IgA. Plates were read at 450 nm after the addition of 3,3′,5,5′-tetrarmethylbenzidine. Antibody titres were calculated using a standard curve that was determined from the reference serum using the calculation software SPECTRA MAX 250 (Molecular Devices).

Adoptive transfer of spleen cells. Spleen cells were obtained from 6-week-old C3H/HeJ (H₂-K¹) mice, and 5 × 10⁶ cells in 0.2 ml were transferred intravenously into SAM-P1 (H₂-K¹) mice immediately after they had been infected intranasally with an inoculum dose of 2.0 × 10⁶ TCID₅₀ RSV per mouse.

Statistical significance. The two-tailed Mann–Whitney U-test and Student’s t-test were used to determine whether a significant difference (P<0.05) existed between SAM-P1 and control SAM-R1 mice.

RESULTS

Susceptibility of SAM-P1 mice to RSV infection

SAM mice were inoculated intranasally with 2 × 10⁶ TCID₅₀ RSV per mouse and the time course of weight loss was investigated (Fig. 1). SAM-P1 mice experienced a weight loss of up to 14 % of their pre-infection weight at day 10 after infection. In contrast, the weight loss of SAM-R1 mice was less than 6 %. Recovery from weight loss in SAM-P1 mice was slow and delayed over 20 days after infection, whilst SAM-R1 mice gained the pre-infection weight quickly. The infected SAM-P1 mice showed sluggish movement and anorexia, and became emaciated. No cases of death were observed in either SAM-P1 or SAM-R1 mice under the experimental conditions used.

Viruses growth in the lungs of SAM-P1 mice was significantly higher than in the control mice, with prolonged shedding of the progeny virus, even on day 12 after...
infection (Fig. 2). Control SAM-R1 mice completely cleared the virus from the lungs by day 8. These results suggested that SAM-P1 mice bore some defects in the defence system against RSV infection.

**Cell infiltration in the lung**

Single-cell suspensions were collected from the lungs and cell populations were identified using specific antibodies for cell markers (Table 1). Following virus infection, a large number of cells infiltrated the lungs of SAM-P1 mice and were retained there for a long period of over 12 days after infection, whilst in the control SAM-R1 mice, infiltrated cells rapidly returned to the normal level (Fig. 3). A major population of cells that appeared in SAM-P1 mice was CD16+ /32+ lymphocytes. It should be noted that SAM-P1 mice showed a decreased proportion of lymphocytes and an increased proportion of granulocytes. The proliferation of CD4+ and CD8+ T lymphocytes in response to RSV infection may be deficient in aged mice (Zhang et al., 2002). No significant difference was observed in the number of CD19+ B cells between the two strains of SAM mice (Table 1).

**Specific CTL and NK cell activity of lung parenchymal cells**

The occurrence of local virus-specific CTL and NK cell responses in lung parenchymal cells was investigated (Fig. 4). Induction of a virus-specific CTL response to RSV infection was impaired in SAM-P1 mice compared with that in SAM-R1 mice at each time point indicated (Fig. 4a). NK cell activity in SAM-P1 mice was also confined to a low level (Fig. 4b). Thus, the impaired cellular immunity to RSV infection in the lungs of SAM-P1 mice was inferred to be associated with elevated virus growth and prolonged virus shedding.

**Virus-specific antibody in BAL fluids**

BAL fluids were collected at intervals after RSV infection and virus-specific antibody titres were determined (Fig. 5). The production of mucosal IgA antibody in SAM-P1 mice was restrained when compared with that of SAM-R1 mice (P<0.05) (Fig. 5c). However, no statistical difference was detected in the antibody titres of IgG1 and IgG2a between SAM-P1 and SAM-R1 mice (Fig. 5a, b).

**Cytokine production by lung parenchymal cells**

To investigate RSV-induced local cytokine production, lung parenchymal cells were collected from mice on various days after infection and cultured in vitro for 48 h in the presence of RSV antigens (Table 2). A significantly high titre of IFN-γ, a T-helper type 1 (Th1) cytokine, was detected in the lungs of control SAM-R1 mice, whilst, in SAM-P1 mice, the production of IFN-γ was restrained and delayed until 9 days after infection. Interestingly, SAM-P1 mice produced much higher amounts of IL-4, a Th2 cytokine, after RSV infection. These findings indicated that the local immune responses in SAM-P1 mice appeared to shift towards a Th2-type dominance.

**Immunity by adoptive transfer of spleen cells**

To confirm the association of the delayed clearance of pulmonary virus in SAM-P1 mice with impaired cellular immunity, spleen cells from C3H/HeJ mice were transferred intravenously into SAM-P1 mice immediately after intranasal infection with RSV. Pulmonary virus growth was determined 9 days later (Table 3). The transfer of
immune-competent spleen cells of C3H/HeJ mice effectively eradicated the challenge virus from the lungs. This suggested that cell-mediated immunity plays a critical role in the defence system against RSV infection.

DISCUSSION

In the present study, we investigated the effect of ageing on the local immune responses to primary RSV infection using an aged model mouse of the SAM strain P1. SAM-P1 mice were apparently more susceptible to RSV infection than control SAM-R1 mice, as demonstrated by pronounced pulmonary virus growth and delayed clearance of the progeny virus. It is well known that virus-infected cells are eradicated mainly by the cytocidal action of cellular immune responses. NK cell activity contributes to a rapid termination of virus infection during an early stage of infection and virus-specific CTLs mediate eradication of RSV from the lungs (Munoz et al., 1991). Children with defects in cell-mediated immunity have difficulty eradicating RSV and exhibit severe lung infections, implying that

Table 1. Distribution of cell populations in the lungs of SAM-P1 and SAM-R1 mice

Mice were infected intranasally with RSV at an inoculum dose of $2 \times 10^6$ TCID$_{50}$ per mouse. Lung parenchymal cells were collected at the indicated intervals after infection and analysed by flow cytometry. Data are the means ± SD of results for five mice tested at each time point.

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Time after infection (days)</th>
<th>No. cells ($\times 10^5$) per lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4$^+$</td>
<td>CD8$^+$</td>
</tr>
<tr>
<td>SAM-P1</td>
<td>0</td>
<td>0.8 ± 0.3*</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.8 ± 0.2*</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2.1 ± 0.1*</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>1.7 ± 0.5*</td>
</tr>
<tr>
<td>SAM-R1</td>
<td>0</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>3.1 ± 0.7</td>
</tr>
</tbody>
</table>

*Significant difference compared with corresponding SAM-R1 mice ($P<0.01$).

Fig. 3. Cell infiltration in the lungs of RSV-infected mice. SAM-P1 (filled bars) and control SAM-R1 (open bars) mice were infected intranasally with RSV at an inoculum dose of $2 \times 10^6$ TCID$_{50}$ per mouse. Data are the means ± SD of results for each group of five mice tested. *, Significant difference compared with corresponding SAM-R1 mice ($P<0.05$).

Fig. 4. Cytotoxicity of lung parenchymal cells collected from RSV-infected SAM-P1 (filled bars) and SAM-R1 (open bars) mice. (a) RSV-specific CTL activity. The effector : target (E : T) cell ratio was 50 : 1. (b) NK cell activity on day 3 after infection. Data are the means ± SD of results for five tested mice. *, Significant difference compared with corresponding SAM-R1 mice ($P<0.01$).
the T-cell population is an important immune component in controlling RSV infection (Hall et al., 1986; de Bree et al., 2005). In fact, CTL and NK cells showed low titres in SAM-P1 mice (Fig. 4), and the transfer of immune-competent spleen cells effectively cleared the virus from the lungs of infected mice (Table 3). The deficiency in cellular immunity may be attributable to a lack of clonal expansion of CD8\(^+\) T cells (Table 1). Furthermore, the decreased production of IFN-\(\gamma\), which stimulates virus-specific CD8\(^+\) CTL responses, together with the augmented production of IL-4, also correlates with the observed immune deficiency (Bangham et al., 1985; Maggi et al., 1992; Abbas et al., 1996; Lee et al., 2005). Recent studies have shown that overexpression of IL-4 causes a delay and suppression of development of the virus-specific CTL population (Fischer et al., 1997; Aung & Graham, 2000). Immunization of mice with the RSV M2 vaccine construct expressing IL-4 induces a significant downregulation of the RSV-specific CD8\(^+\) CTL response, as well as of IFN-\(\gamma\) production (Tang & Graham, 1994; Aung et al., 1999). These findings are in concert with our concept that the pronounced production of IL-4 in RSV-infected SAM-P1 mice reduces its local CTL response.

RSV-induced CD4\(^+\) T cells play a role in virus clearance, but they are not the primary effector cells (Plotnick-Gilquin et al., 2000). The reduced number of CD4\(^+\) T cells in the lungs of RSV-infected SAM-P1 mice was due to an early involution of the thymus (Toichi et al., 1997) and a low efficiency of T-cell proliferation in response to RSV antigens (Table 1). Reduced CD4\(^+\) T cells in SAM-P1 mice could cause a weak response of local IgA antibody production (Fig. 5c). Specific antibodies to the attachment (G) and fusion (F) virus envelope proteins are potentially protective (Glezen et al., 1981; Walsh & Falsey, 2004) and contribute to elimination of the progeny virus at a later phase of infection, probably through antibody-dependent immune cytolysis (Hashimoto et al., 1983; Falsey et al., 1999). In particular, the IgA subclass antibody in the upper respiratory tract is important for protection against viruses

**Table 2.** Local cytokine production in the lungs of SAM-P1 and SAM-R1 mice

Mice were infected intranasally with RSV at an inoculum dose of \(2 \times 10^6\) TCID\(_{50}\) per mouse. Lung parenchymal cells were collected at the indicated intervals after infection and restimulated *in vitro* with UV-inactivated RSV. The supernatants were collected 48 h after incubation and assayed for cytokine production. Data are the means ± SD of results for five mice tested at each time point.

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Time after infection (days)</th>
<th>Cytokine titre (pg ml(^{-1}))</th>
<th>IL-4</th>
<th>IFN-(\gamma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAM-P1</td>
<td>0</td>
<td>(&lt;5.0)</td>
<td>(&lt;5.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>(&lt;5.0)*</td>
<td>(&lt;5.0)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>86.5 ± 23.6*</td>
<td>(&lt;5.0)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>128.7 ± 4.2*</td>
<td>12.7 ± 4.5*</td>
<td></td>
</tr>
<tr>
<td>SAM-R1</td>
<td>0</td>
<td>(&lt;5.0)</td>
<td>(&lt;5.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>(&lt;5.0)</td>
<td>10.9 ± 2.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>20.8 ± 16.7</td>
<td>14.8 ± 2.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>70.2 ± 11.6</td>
<td>51.4 ± 6.1</td>
<td></td>
</tr>
</tbody>
</table>

*Significant difference compared with corresponding SAM-R1 mice (\(P<0.01\)).

**Table 3.** Immunity of SAM-P1 mice by adoptive transfer of immune-competent spleen cells

Normal spleen cells (\(5.0 \times 10^7\)) were transferred intravenously into SAM-P1 mice immediately after intranasal infection with RSV. Lung homogenates were collected on day 9 after challenge infection and assayed for infectivity. Data are the means ± SD of results for five tested mice.

<table>
<thead>
<tr>
<th>Mouse spleen cells</th>
<th>Pulmonary virus titre (log TCID(_{50}) per lung)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H/HeJ</td>
<td>(&lt;2.0)*</td>
</tr>
<tr>
<td>None</td>
<td>3.6 ± 0.5</td>
</tr>
</tbody>
</table>

*Significant difference compared with corresponding non-transferred mice (\(P<0.01\)).
such as RSV that cause surface infection (Renegar & Small, 1991). The ability of Th1 and Th2 cells to stimulate the production of IgG subclass antibodies was not altered in SAM-P1 mice (Fig. 5a, b).

Pneumonia is characterized by the recruitment of inflammatory cells, mainly granulocytes, to the local site of infection (Skerrett, 1994). The influx of granulocytes into the lung alveolar compartment during RSV infection was markedly increased in SAM-P1 mice (Table 1). The weight loss observed in SAM-P1 mice was consistent with the increased cell infiltration in the lungs (Figs 1 and 3). It is conceivable that the vigorous recruitment of granulocytes, especially at day 3 after infection, was induced by abundant and prolonged virus loading in the lungs (Fig. 2) and triggered the increased morbidity. A significant weight loss with high-titre virus replication in the lung has been observed in old BALB/c mice following infection with a high-inoculum dose of RSV (Graham et al., 1988). Age-dependent RSV replication also occurs in the cotton rat (Curtis et al., 2002). These findings suggest that the inoculum dose of challenge virus and the age of the mouse may be important factors for RSV-induced illness. We suggest that the early appearance of peak virus titre in SAM-P1 mice at day 3 of infection is related to the high-inoculum dose of RSV and the specific strain of mouse used (Fig. 2).

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