Bronchoalveolar lavage cell analysis in measles viral pneumonia


ABSTRACT: Although the immunological changes due to measles virus infection, such as suppression of delayed skin reactivity and increase in soluble CD8 in peripheral blood, have been demonstrated, the immunological changes in the lung during measles viral pneumonia (MVP) have not been reported. The aim of this study was to clarify the intrapulmonary immunological changes in MVP.

We analysed cell differentials and lymphocyte surface antigens of bronchoalveolar lavage (BAL) cells, both in the acute and the convalescent phase of MVP by flow-cytometry using CD4+, CD8+, CD8+CD11b+ and CD8+CD8- mononuclear antibodies in 10 patients and six healthy control subjects. The absolute numbers of CD8+ and CD8+CD11b- cells were significantly greater both in the acute and the convalescent phase compared with those in controls. The percentages of CD8+ and CD8+CD11b- cells in the acute phase were significantly greater than those in controls and the convalescent phase. The CD4/CD8 ratios in the acute phase were significantly smaller than those in the convalescent phase and controls.

In conclusion, the intrapulmonary immunological changes with an increase in CD8+ and CD8+CD11b- cells in BAL fluid of MVP are thought to be the primary reaction in measles virus-infected lungs.

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Measles is still a common illness in the world [1, 2]. The most common complication of this disease is pneumonia which is classified as primary measles viral pneumonia (MVP) or secondary bacterial pneumonia [3, 4].

A number of studies have demonstrated the immunological changes due to measles virus infection. These changes include the suppression of delayed skin reactivity [5-9] and abnormally low mitogen responses [10, 11]. In peripheral blood, spontaneous proliferation of mononuclear cells [5], and increases in soluble CD8 [12], soluble interleukin-2 receptor [12], and gamma-interferon production [13] have been described.

Human T-lymphocyte subpopulations have been defined, as CD4+ (helper/inducer) and CD8+ (suppressor/cytotoxic) cells by using monoclonal antibodies [14-17]. Furthermore, the functional characteristics of two-colour-defined T-cells have been examined by the sequential cell sorter technique [18]. It has been reported that CD8+CD11b- and CD8+CD11b+ cells show suppressor [19, 20] and cytotoxic [21, 22] activity, respectively. In addition, increased proportions of the suppressor/cytotoxic cell phenotype have been demonstrated in cytomegalovirus [23], and hepatitis B virus [24] infections, suggesting that such a change in T-lymphocyte subpopulations might be a general phenomenon in viral infections [25].

To our knowledge, there has been no study on the immunological changes in the lung during MVP. The present study was designed to clarify the intrapulmonary immunological changes in MVP. We analysed cell differentials and lymphocyte surface antigens of bronchoalveolar lavage (BAL) cells, both in the acute and the convalescent phase of MVP, by flow-cytometry using anti-CD4, anti-CD8 and anti-CD11b antibodies.

Subjects

Five patients with measles, 14-20 yrs of age, were admitted to Asanogawa General Hospital, from April 15 to June 9, 1991 (table 1). The diagnosis of measles was established by a history of a prodrome and physical signs including macropapular rash, conjunctivitis, Koplik's spots and elevated immunoglobulin M (IgM) antibody level to measles virus in the serum by enzyme immunoassay. The diagnosis of coexisting MVP was made, based on the following findings: 1) dry cough; 2) decrease in arterial oxygen pressure (increase in alveolar-arterial oxygen pressure gradient); 3) no increase in white blood cell counts in peripheral blood; 4) no detection of bacteria, mycobacteria or fungus from BAL fluid (BALF);
Table 1. Clinical features of patients with measles viral pneumonia

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>WBC (cells/μl)</th>
<th>PaO₂ (torr/kPa)</th>
<th>PaCO₂ (torr/kPa)</th>
<th>FVC % pred</th>
<th>FEV₁ % pred</th>
<th>FEV₁/FVC %</th>
<th>Days after rash onset</th>
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<tbody>
<tr>
<td>Acute phase</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>20</td>
<td>M</td>
<td>2100</td>
<td>68.8/9.2</td>
<td>36.8/4.9</td>
<td>95/77</td>
<td>78/4</td>
<td>81</td>
<td>4</td>
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<tr>
<td>2</td>
<td>14</td>
<td>F</td>
<td>3200</td>
<td>73.8/9.8</td>
<td>37.7/5.0</td>
<td>99/93</td>
<td>90/5</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>F</td>
<td>3500</td>
<td>83.2/11.1</td>
<td>43.7/5.8</td>
<td>ND/114</td>
<td>85/74</td>
<td>75</td>
<td></td>
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<tr>
<td>4</td>
<td>20</td>
<td>M</td>
<td>5200</td>
<td>76.8/10.2</td>
<td>43.7/5.8</td>
<td>ND/114</td>
<td>85/74</td>
<td>75</td>
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</tr>
<tr>
<td>5</td>
<td>18</td>
<td>M</td>
<td>5400</td>
<td>73.3/9.8</td>
<td>45.9/6.1</td>
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<td>88/83</td>
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</tr>
<tr>
<td>Mean±SEM</td>
<td>3900±600</td>
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<td>75.2±2.4</td>
<td>41.8±2.0</td>
<td>5.6±0.3</td>
<td>101±4.5</td>
<td>86±3.3</td>
<td>90±8</td>
<td>6.8±1.6</td>
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<td>Convalescent phase</td>
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<tr>
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<td>81/29</td>
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<tr>
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<td>3800</td>
<td></td>
<td>92.2/12.3</td>
<td>48.1/6.4</td>
<td>122/116</td>
<td>90/30</td>
<td>30</td>
<td>30</td>
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<tr>
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<td>111/94</td>
<td>84/37</td>
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<td>4</td>
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<td>96.2/12.8</td>
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<td>48.2/6.4</td>
<td>106/89</td>
<td>83/27</td>
<td>67</td>
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<tr>
<td>Mean±SEM</td>
<td>5300±500</td>
<td></td>
<td>97.5±1.5</td>
<td>44.1±2.2</td>
<td>5.9±0.3</td>
<td>108±5.9</td>
<td>95±7.8</td>
<td>84±2.2</td>
<td>46.0±8.7*</td>
</tr>
</tbody>
</table>

*: p<0.01; **: p<0.005; compared with acute phase. WBC: white blood cells; PaO₂: arterial oxygen tension; PaCO₂: arterial carbon dioxide tension; FVC: forced vital capacity; FEV₁: forced expiratory volume in one second; ND: not determined.

5) linear-reticular infiltrate on chest roentgenogram (fig. 1); and 6) histologically documented alveolitis (fig. 2) on transbronchial lung biopsy specimens. The control population consisted of six healthy volunteers without history of previous pulmonary disease. All subjects were lifetime nonsmokers. Informed consent was obtained from all the subjects and their families. This study was approved by the Ethics Committee of our hospital.

Methods

Procedure of bronchoalveolar lavage

Fibreoptic bronchoscopy was performed through the transoral route, after topical anaesthesia with 2% lidocaine. Premedication with atropine (0.5 mg) and hydroxyzine pamoate (25 mg) was routinely performed, and a further sedation with 2.5–5.0 mg of intravenous diazepam was given when required. The tip of the instrument was advanced and wedged into a segmental or subsegmental bronchus in the middle or lingular lobe. Sterile 0.9% saline solution was then infused in 50 ml aliquots to a total of 150 ml. The fluid was recovered by gentle aspiration after each aliquot. The volume of fluid recovered was always above 50% of fluid instilled.

The procedure of BAL was performed in both the acute (6.8±1.6 days after the onset of the rash) and the convalescent (46.0±8.7 days after the onset of the rash) phases of MVP.

Analysis of bronchoalveolar lavage cells

Total cell number and differentials. Cellular analyses were carried out as described previously [26]. After filtering the fluid through a double layer of Dacron nets, cells were centrifuged at 1,500 rpm for 10 min and resuspended in 10 ml of RPMI-1640 (Grand Island Biological Co., Grand Island, NY, USA). Following dilution with...
an equal volume of Turk solution, the number of cells was counted in a Bürker chamber. The fluid was then resuspended with RPMI-1640 to make a cell suspension containing $2 \times 10^6$ cells/ml. Smears for differential counts of the cells were prepared by cytocentrifugation (Cytospin Shandon 2) at 1,000 rpm for 10 min. After staining with May-Grünwald-Giemsa stain, 300 cells were counted.

**Lymphocyte subsets.** The cell suspension was adjusted to a concentration of $2 \times 10^6$ cells/ml. Then, 10 µl of monoclonal antibody at the proper dilution was added to 100 µl of this cell suspension. The cells were incubated at 4°C for 30 min in the dark, washed twice in phosphate-buffered saline, and subsequently resuspended in 0.3 ml of RPMI-1640. The suspended cells were then analysed for CD4+, CD8+, CD8+CD11b+ and CD8+CD11b- lymphocytes, using a flow cytometer (FACScan, Becton Dickinson). Monoclonal antibodies recognizing CD8 (fluorescein isothiocyanate (FITC)-conjugated Leu2), CD4 (FITC-conjugated Leu3), CD11b (phycoerythrin (PE)-conjugated Leu15) and determinants were provided by Becton Dickinson. In each analysis, cells stained by FITC- and PE-conjugated nonreactive mouse IgG (Becton Dickinson) were employed as negative control. Flow cytometric analyses were processed by SimulSET software in a FACScan. Appropriate fractions of lymphocyte were selected by gating on two dimension display, on forward scatter and side scatter.

**Statistical analysis**

Data were expressed as mean±SEM. Differences between the values in the acute and the convalescent phases of MVP were analysed by the Wilcoxon signed-rank test. Differences between the patients with MVP and the control subjects were analysed by the Mann-Whitney U-test. A p value of $\leq 0.05$ was considered to be significant.

**Results**

**Total cells and differentials**

The total cell number in BALF obtained from the patients in the acute phase of MVP was significantly greater than that in the control subjects ($p<0.05$) (fig. 3).

The percentage of macrophages in both the acute and the convalescent phase was smaller than that in the control subjects ($p<0.02$ and $p<0.05$, respectively). On the other hand, there was no significant difference in the absolute number between the three groups (fig. 3).

The lymphocyte population in both the acute and the convalescent phase of MVP was significantly greater than that in the control subjects, both in percentage ($p<0.02$ and $p<0.05$, respectively) and in absolute number ($p<0.01$ and $p<0.02$, respectively) (fig. 3).

The percentage of neutrophils in the acute phase of MVP was significantly greater than that in the convalescent phase of MVP ($p<0.02$). The absolute number of neutrophils in the acute phase was significantly greater than that in the control subjects ($p<0.05$) (fig. 3).

There was no significant difference in the percentage or the absolute number of eosinophils between the three groups (fig. 3).

**Lymphocyte subsets and CD4/CD8 ratio**

The percentage of CD4+ cells was significantly smaller in the acute phase of MVP than in the control subjects and the patients with the convalescent phase of MVP ($p<0.05$ and $p<0.01$, respectively). However, there were no significant differences between them in the absolute number (fig. 4).

Figure 5 is an example of expression of CD8 and CD11b by lymphocytes obtained from BALF in a two-colour FACScan analysis (patient No. 1). The
percentages of CD8+ and CD8+CD11b- cells in the patients in the acute phase were significantly greater than those in the patients in the convalescent phase and in the control subjects (p<0.05 and p<0.01, respectively). The absolute numbers of CD8+ and CD8+CD11b- cells both in the acute and the convalescent phase were significantly greater than those in the control subjects (p<0.01 and p<0.01, respectively) (fig. 4).

The absolute number of CD8+CD11b+ cells in the patients in the acute and the convalescent phase of MVP was significantly greater than those in the control subjects (p<0.01 and p<0.05, respectively). However, the percentages were no significantly different (fig. 4).

The CD4/CD8 ratio in the acute phase of MVP was significantly less than in the convalescent phase and in the control subjects (p<0.05 and p<0.05, respectively) (fig. 6).

**Discussion**

Primary measles virus infection is immunologically characterized both by the development of a strong anti-viral immune response [27, 28] and multiple abnormalities of immune regulation [5–11]. Regarding the anti-viral response, Besh [27] reported the development
of complement fixing antibodies in measles patients. Graves et al. [28] showed that large amounts of antibody to nucleocapsid protein of measles virus developed in the patients by day one of the rash. Concerning abnormalities of immune regulation, it has been well established that skin test reaction to tuberculin decreases during measles infection [5-9], that lymphoproliferative responses to mitogens are depressed in measles patients [10, 11]. On the other hand, in spite of lymphocytopenia, no change in the ratio of helper T-lymphocytes to suppressor cytotoxic cells has been shown in peripheral blood of measles patients [11, 29, 30].

Measles is often complicated by primary or secondary pneumonia, otitis media, encephalitis, for example [1-4]. Although it has been reported that soluble CD8 increases in cerebrospinal fluid of measles patients with encephalomyelitis [12], it has never been determined whether inflammatory cells and lymphocyte subpopulation in the lungs change in MVP. Therefore, we examined bronchoalveolar lavage cells in five patients with MVP and compared the data between the acute and the convalescent phases.

Our study showed that lymphocytes in BALF significantly increased in parallel with the increment in CD8+ and CD8+CD11b- cells, which resulted in a decreased CD4+/CD8 ratio, in the acute phase of MVP. CD8 is a 33-kDa transmembrane protein, present as an associative recognition molecule in the CD3+ cell receptor complex on the cytotoxic or suppressor subpopulation of T cells [31, 32]. The interaction of the CD8+ cell with a target cell bearing histocompatibility locus antigens (HLA) Class I [33], such as a virus-infected cell, results in the activation of the CD8+ cell and phosphorylation of CD8 coincidentally with the cytotoxic response [34]. The precursor and effector cytotoxic T-lymphocytes express the CD8+CD11b- phenotype [21]. It has been suggested that CD8+ and CD8+CD11b- cells may be important in the clearance of measles virus from the lungs in the acute phase of the pneumonia. This reaction seems to be a primary, but not a secondary, reaction in the infected lungs, because it has been shown that CD8+ cells in the peripheral blood do not increase in measles patients with or without pneumonia [11, 29, 30].

However, McFarland et al. [35] have recently reported that CD8+ cells are cytotoxic memory cells, and that the percentage of CD8+CD11b- splenocytes in mice gradually increased to a maximum level of expression at 1-2 days after lymphocyte choriomeningitis virus infection, and then declined to near naive levels (<3% of naive mouse splenocytes) by day 19-20 postinfection. In our study, in contrast to CD8+CD11b- cells, the percentage of CD8+CD11b+ cells did not significantly increase. The main reason for this discrepancy between CD8+CD11b- cells and CD8+CD11b+ cells may be that we performed BAL too late to observe the increase in the percentage of CD8+CD11b+ cells. BAL in the acute phase of MVP was performed at 6.8±1.6 days after the onset of the rash. Since the time from the virus exposure to the appearance of rash is about 2 weeks [36],


