Early Augmentation of Interleukin (IL)-12 Level in the Airway of Mice Administered Orally with Clarithromycin or Intranasally with IL-12 Results in Alleviation of Influenza Infection

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ABSTRACT

The protective role of interleukin (IL)-12 against influenza infection was assessed by analyzing the efficacies of orally administered clarithromycin (CAM) as an immunomodulator and intranasal administration of recombinant IL-12 in intranasally influenza virus-infected mice. In infected mice, CAM at 20 mg/mouse/day significantly elevated the levels of IL-12 and interferon-γ on days 2 and 3, respectively, after infection in the bronchoalveolar lavage fluid (BALF), but the levels in the sera were not affected. The levels of IL-4, -6, and -10 were not significantly affected in the sera and BALF. Corresponding with the local elevation of IL-12 level, CAM reduced virus yield and the number of infiltrated cells in the BALF, the severity of pneumonia, and mortality of the treated mice. The potential activity of CAM as an experimental immunomodulator was verified at a dose of 20 mg/mouse/day. Intranasal administration of the optimal dose (20 ng/mouse) of IL-12 on day 2 significantly reduced virus yield in the BALF after infection. The loss of body weight was significantly suppressed by IL-12 administration. The local elevation of IL-12 level at the optimal dose and timing in influenza infection was confirmed to be effective in alleviating the influenza infection in mice treated with the two different ways. Thus, the augmentation of IL-12 production or administration of supplementary IL-12 in the respiratory tract was essential in reducing virus yield in the early phase of influenza and may be crucial for recovery from influenza infection.

Efficacy of macrolide administration has been demonstrated in diffuse panbronchiolitis (Oda et al., 1995). This therapy is also effective on sino-bronchial syndrome and chronic sinusitis (Iino et al., 1993). The pivotal mode of action in the therapy is indicated not to be bactericidal. Among macrolides, FK506 has been shown to selectively bind to a cellular receptor and control immune responses as an immunosuppressant (Harding et al., 1989; Liu et al., 1991). Erythromycin, by inhibiting the induction of interferon (IFN)-γ, has substantial therapeutic value for influenza virus-induced pneumonia by inhibiting the inflammatory-cell response (Sato et al., 1998). A variety of activities of macrolide antibiotics have been reported on immunocompetent cells, inflammatory cells, and epithelial cells (Keicho et al., 1994; Oda et al., 1994; Suzuki et al., 1997). Clarithromycin (CAM) is a macrolide antibiotic and a derivative of erythromycin and has been used for treating chronic lower respiratory tract infections. The spectrum of CAM-antibacterial activity is broader than that of erythromycin. Recently, CAM has been shown to alter cytokine production in human macrocytes and possess immunomodulatory activity in vitro (Khan et al., 1999). It may play a role, in vivo, not only as an antibacterial drug but also as an immunomodulator.

Intranasal influenza virus infection models in mice have been widely used to evaluate the pathogenic or biological roles of cytokines in influenza infection. In a murine infection model, cytokines, e.g., interleukin (IL)-12, IFN-γ, IL-4, IL-10, IL-1α, IL-1β, IL-6, and tumor necrosis factor-α are produced both locally and systemically (Hennet et al., 1992; Conn et al., 1995; Kurokawa et al., 1996a,b; Monteiro et al., 1998). The production of various cytokines is time-dependent, and after influenza infection, it may contribute to the development and activation of an immune response against influenza in the early phase of infection, possibly resulting in a reduction of virus yield in the respiratory tract. Also, in this murine model, the development of pneumonia is well characterized in the late phase of infection. The pathologic changes are similar to those seen in humans (Tashiro et al.,

ABBREVIATIONS: IFN, interferon; ANOVA, analysis of variance; BALF, bronchoalveolar lavage fluid; CAM, clarithromycin; CC50, the concentration of CAM reducing cell viability by 50%; CTL, cytotoxic T lymphocyte; IL, interleukin; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline; PFU, plaque-forming units; NO, nitric oxide; ELISA, enzyme-linked immunosorbent assay.
1987; Kurokawa et al., 1998), based on infiltration of immune cells into the lung following the early immune response after influenza infection (Oda et al., 1989; Akaike et al., 1996). Thus, this murine model is useful to evaluate and analyze the effects of CAM, especially as an immunomodulator.

Monteiro et al. (1998) demonstrated an important role of IL-12 for protective efficacy in influenza infection by using anti-IL-12 antibody. In this study, we have focused on the effects of augmentation of IL-12 production by CAM and intranasal administration of IL-12, which may contribute to the early development of innate immune response on influenza infection. We have clarified the protective role of IL-12 production in the respiratory tract in the early phase of influenza infection by the augmentation of IL-12 production mediated by CAM and intranasal administration of IL-12.

**Materials and Methods**

**Cells and Viruses.** Madin-Darby canine kidney (MDCK) cells were grown and maintained in Eagle’s minimum essential medium, supplemented with 5 and 2% heat-inactivated calf serum, respectively. Mouse adapted influenza virus [A/PR/8/34(H1N1)] was propagated in the lungs of mice by intranasal infection (Kurokawa et al., 1990). The lungs of infected mice were removed and homogenated in phosphate-buffered saline (PBS). The homogenate was centrifuged at 3000 rpm for 15 min, and then the supernatant was stored at −80°C.

**Compounds.** CAM was supplied by Taisyo Pharmaceutical Co., Ltd. (Tokyo, Japan) and suspended in 5% arabic gum at 0, 5, and 50 mg/ml. Each suspension of 0.2 ml was orally administered to mice. For in vitro assays, CAM was dissolved in ethanol at 5% and diluted with culture medium to make its various final concentrations, as described below. The concentration of ethanol in each culture medium was less than 0.5%. Recombinant mouse IL-12 was purchased from Pepro Tech EC Ltd. (London, England) and dissolved in pyrogen-free saline for intranasal administration to infected mice.

**Animals.** Female DBA/2 Cr mice (7-week-old, 19–21 g) were purchased from Sankyo Labo Service Co., Ltd., Tokyo, Japan. The mice were housed five per cage in specific pathogen-free conditions with food and water ad libitum and under a 12-h light/dark diurnal cycle (light at 7:00 AM). The temperature in the room was kept at 23 ± 2°C. The mice were acclimated for at least 3 to 4 days before starting experimental procedures. The animal experimentation guidelines of Toyama Medical and Pharmaceutical University (Toyama, Japan) were followed in the animal studies.

**Murine Influenza Virus Infection Model.** DBA/2 mice were intranasally infected or mock-infected with influenza virus at 1500 plaque-forming units (PFU)/25 μl of PBS under ether anesthesia (Kurokawa et al., 1996a, 1998). CAM was orally administered to the mice by a gavage, two times daily for 7 days at approximately 12-h intervals at doses of 0, 1, and 10 mg/mouse, starting a day before infection. Each group contained 10 mice. The body weights of 10 mice in each group were measured every morning after infection. The changes of body weight were calculated based on the body weight of each mouse on day 0. To determine their mortality, the infected mice were fed and observed for at least half a month after infection.

**Preparation of Infiltrated Cells.** The number of infiltrated cells in the BALF of lungs was examined on days 1 to 4 after infection. The BALF was obtained by instilling 1 ml of Eagle’s minimum essential medium into the lungs and aspirating it from the trachea of mice using a tracheal cannula three times, as described previously (Kurokawa et al., 1996a). The BALF was prepared from four to five mice in each group. The cells infiltrated in the BALF were collected by centrifugation (3000 rpm for 10 min), and the number of live cells in the infiltrates of BALF was determined by a trypan blue exclusion test. The supernatant was stored at −80°C for the determination of cytokine levels and virus yield.

**Histological Analysis of Lungs.** Lungs were resected and examined histologically in untreated and CAM-treated mice. On days 3 and 5 after infection, the resected lungs were fixed with 5% paraformaldehyde solution. The specimens were dehydrated and embedded in paraffin. Four-micrometer thick sections were cut and stained with hematoxylin and eosin. These paraffin sections were photographed with a digital camera, and the pictures were printed with a magnification of 10. The lung tissue areas and the inflammatory areas were outlined on each picture. Using a Nikon Cosmozone Morphometric System (Nikon, Tokyo, Japan), the lung and inflammatory areas were measured by tracing their outlines on the digitizer. The percentages of area of pneumonia in the total area of lungs observed were determined (Nagasaka et al., 1995; Kurokawa et al., 1996b).

**Determination of Virus Yield in BALF.** Virus yields in the BALF of influenza virus-infected mice were examined on days 1 to 4 after infection. BALF was prepared as described above. Virus titers of stored supernatant of BALF were determined by the plaque assay using MDCK cells, as described previously (Kurokawa et al., 1990).

**Plaque Reduction Assay.** CAM was examined for its anti-influenza virus activity in the plaque reduction assay to estimate the possible anti-influenza activity in vivo. Duplicate cultures of MDCK cells in 60-mm plastic dishes were infected with 100 PFU/ml of PR8 strain for 1 h at room temperature. Cells were overlaid with 5 ml of nutrient agarose (0.8%) medium containing various concentrations of CAM and then cultured at 37°C for 2 to 3 days. The infected cells were fixed with 5% formalin solution and stained with 0.03% methylene blue solution. The number of plaques was counted under a dissecting microscope (Kurokawa et al., 1990). The EC50 values for plaque reduction were determined from a curve relating the plaque number to the concentrations of CAM.

To examine the effects of pretreatment of influenza virus with CAM, 1000 PFU/ml of PR8 strain was mixed with an equal volume of CAM at various concentrations for 1 h at 37°C. Viruses in the mixture (100 PFU/0.2 ml) were adsorbed to MDCK cells in 60-mm plastic dishes for 1 h at 37°C, and then cells were rinsed three times with PBS. The rinsed cells were overlaid with 5 ml of nutrient agarose (0.8%) medium for plaque formation, and then the number of plaques and EC50 values for plaque reduction were determined as described above.

**Cytotoxicity Assay.** Cytotoxicity of CAM was examined by the growth inhibition of MDCK cells to evaluate its direct anti-influenza virus activity properly. Briefly, MDCK cells were seeded at a concentration of 2.5 × 104 cells/well in 24-well plates and grown at 37°C for 2 days. The culture medium was replaced with fresh medium containing CAM at various concentrations, and cells were further grown for 2 days. The cells were treated with trypsin, and the number of viable cells was determined by a trypsin blue exclusion test. The concentration of CAM reducing cell viability by 50% (CC50) was determined from a curve relating the percentage of cell viability to the concentrations of CAM.

Cytotoxicity for the pretreatment with CAM was examined against MDCK cells. The cells were grown at 37°C for 2 days as described above and treated with CAM (0.2 ml) at various concentrations for 1 h at 37°C. The cells were rinsed three times with PBS and grown for 2 days, and then the number of viable cells was determined by a trypsin blue exclusion test.

**Determination of Cytokine-Levels in BALF and Serum.** The levels of cytokines (IL-12, IFN-γ, IL-4, IL-6, and IL-10) were examined in the BALF and serum on days 1 to 4 after infection. The levels of IL-12 were also examined in the BALF and serum of mock-infected mice administered with CAM at 0 or 20 mg/day on day 2. Blood was taken from four to five mice in each group. BALF was prepared as described above. Cytokine levels in the BALF and serum were determined using ELISA kits (Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Buckinghamshire, UK; BioSource International, Inc., Camarillo, CA) according to the manufacturer’s instructions.
The level of IL-12 was measured as that of IL-12 p70, which is biologically active.

**Intranusal IL-12 Administration to Infected Mice.** The effect of IL-12 on influenza infection was examined in mice. Mice were infected with influenza virus at 100 PFU/20 μl/mouse as described above. The infected mice were intranasally administered with IL-12 at 20, 100, and 500 mg/20 μl/mouse, once on days 0 to 3 after infection. Control mice received 20 μl of pyrogen-free saline. The body weight of each mouse was monitored daily for 7 days after infection. On days 2 to 5 after infection, BALF was prepared and virus yields in the BALF were determined as described above.

**Statistical Analysis.** The one-way analysis of variance (ANOVA) followed by a post hoc Dunnett's test was used to evaluate the statistical significance of differences between two groups in the areas of pneumonia, the concentrations of CAM for in vitro assay, and the concentrations of cytokines. The repeated measure two-way ANOVA was used to analyze the interaction between a treated group and a control in the changes of net body weights, virus yields, the levels of cytokines, and the number of infiltrated cells. Statistical differences in the mortality were evaluated using the Kaplan-Meier method. A p value of less than 0.05 was defined as statistically significant.

### Results

**Experimental Efficacy of CAM.** The efficacy of CAM was evaluated in an intranasal influenza virus infection model in mice using 2 and 20 mg/day of CAM. The former dose was used as a dose for mice corresponding to a human dose, and the latter dose was used as the higher dose to assess the potential activity of CAM as an experimental immunomodulator. Since the 50% lethal dose of CAM was 2700 mg/kg/day for mice (Abe et al., 1988), the 2- and 20-mg/day doses were used as nontoxic doses. As shown in Fig. 1, 20 mg/day of CAM significantly reduced the mortality of infected mice (p < 0.05 by the Kaplan-Meier method) but was ineffective at 2 mg/day. The body weights of control mice decreased markedly 2 days after infection (Fig. 2A). However, 20 mg/day of CAM significantly reduced the loss of body weight (Fig. 2A, p < 0.01 by repeated measure two-way ANOVA), although 2 mg/day of CAM again showed no significant effect. In this experiment, we analyzed the statistical significance of body weight loss for only 4 days after infection, as the loss of infected mice due to death did not permit statistical analysis. Histopathological analysis of the inflammatory areas of lungs showed that 20 mg/day of CAM retarded the development of pneumonia in infected mice on days 3 and 5 after infection (Table 1, p < 0.05 by one-way ANOVA on day 5). When the number of infiltrated cells into the BALF was examined 1 to 4 days after infection, 20 mg/day of CAM significantly reduced the number of infiltrated cells (Fig. 2B, p < 0.05 by repeated measure two-way ANOVA). This was consistent with the alleviation of pneumonia in mice treated with 20 mg/day of CAM. The reduction of infiltrated cells in the BALF, the alleviation of pneumonia, and the reduction in mortality were confirmed in infected mice treated with 20 mg/day of CAM by repeating the experiments. CAM of 20 mg/day showed significant experimental efficacy in influenza virus-infected mice, but 2 mg/day had no effect.

**Virus Yield in BALF of Infected Mice.** CAM at 20 mg/day significantly reduced virus yields in the BALF of infected mice compared with controls (p < 0.05 by repeated measure two-way ANOVA; Fig. 2C) but 2 mg/day was ineffective. CAM at 20 mg/day suppressed virus growth in the respiratory tract and exhibited anti-influenza virus activity in mice.

**Anti-Influenza Virus Activity in Vitro.** CAM was examined for its anti-influenza virus activity in the plaque reduction assay using MDCK cells to evaluate whether the suppression of virus growth in the respiratory tract of mice was due to the direct anti-influenza virus activity of CAM. As shown in Table 2, the EC_{50} (39.3 ± 6.6 μg/ml) and CC_{50} (44.5 ± 7.7 μg/ml) values of CAM added after virus absorption to cells were not statistically significant. Furthermore, we evaluated the direct anti-influenza virus activity of pretreatment with CAM in the influenza virus-infected mice. When we examined the effect of CAM on the attachment of virus to cells by the pretreatment of virus with CAM, there was no significant difference between the EC_{50} and CC_{50} values. Thus, CAM did not exhibit any anti-influenza virus activity in following either pre- or post-treatment in vitro. The suppression of virus yield in BALF of mice administered with CAM, before and after influenza virus infection, seems to be independent of a direct anti-influenza virus activity of CAM.

**Effect of CAM on Production of Cytokines in Infected Mice.** CAM significantly reduced mortality and alleviated influenza symptoms in mice (Figs. 1 and 2). To evaluate the activity of CAM as an immunomodulator, the effects of CAM at 2 and 20 mg/day on cytokine production were measured to assess its effects as an immune mediator in influenza infection using influenza virus-infected mice on days 1 to 4 after infection. Levels of IL-12, an inducer of Th1 immune response, IFN-γ, a Th1 cytokine, IL-4 and IL-10, Th2 cytokines, and IL-6, an inflammatory cytokine were measured in the BALF and sera. Levels of IL-12 in the BALF were significantly elevated by the treatment with CAM at 20 mg/day after infection (Fig. 3A, p < 0.05 by repeated measure two-way ANOVA). On day 2 after infection, this elevation was significantly changed (Fig. 3A, p < 0.05 by one-way ANOVA). In mock-infected mice on day 2, no significant elevation of IL-12 levels was seen following administration of CAM at 20 mg/kg compared with controls (Fig. 3A). CAM at 20 mg/day also significantly increased the level of IFN-γ in the BALF on day 3 (Fig. 3E, p < 0.05 by one-way ANOVA). Elevations of IL-12 and IFN-γ levels were not seen in the sera of infected mice treated with CAM at 20 mg/day (data not shown). The levels of other cytokines (IL-10, IL-4, and IL-6) examined in the BALF and sera were unchanged by CAM.

![Fig. 1. Effect of CAM on the survival rate of influenza virus-infected mice. CAM at 20 mg/day was orally administered to influenza virus-infected mice as described in text. Ten mice were used in each group. *p < 0.05 versus no CAM control by the Kaplan-Meier method for 8 days after infection.](attachment:image.png)
Effect of Intranasal Administration of IL-12 in Infected Mice. We examined the change of body weight of infected mice given recombinant IL-12 (100 ng/mouse) intranasally once at various days after infection. On day 2, IL-12 administration was more effective in suppressing loss of body weight compared with days 0, 1, and 3 after infection (data not shown). This result was consistent with an augmentation of IL-12 in CAM-treated mice at 2 days after infection as described above. Furthermore, we found that IL-12 levels at 20 ng/mouse suppressed the loss of body weight of infected mice, but it was ineffective at 100 and 500 ng/mouse (Fig. 4A, p < 0.05 by repeated measure two-way ANOVA). Intranasal IL-12 (20 ng/mouse) was an optimal dose for the suppression of the weight loss and also decreased virus yield when given once on day 2 after infection (Fig. 4B, p < 0.05 by repeated measure two-way ANOVA). Therefore, intranasal administration of IL-12 was effective in reducing the virus yield of the influenza virus in the BALF and alleviating influenza.

**Discussion**

In this study, the protective role of IL-12 against influenza infection was evaluated by assessing the efficacies of oral administration of CAM and intranasal administration of IL-12 in influenza virus-infected mice. Thus, local elevation of IL-12 levels in the respiratory tract were found to be effective in reducing the virus yield of influenza virus in the BALF and alleviating the symptoms of influenza in mice. When we examined the efficacy of CAM at 20 mg/day in influenza virus-infected mice, CAM augmented local IL-12 production followed by IFN-γ production and was effective in producing recovery from influenza infection. This dose of CAM was used to evaluate the activity of CAM as an experimental immunomodulator in mice, although the dose of CAM at 20 mg/day was higher than a dose for mice, corresponding to a human dose that has been used for the treat-

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**TABLE 1**

Effect of CAM on the development of pneumonia in influenza virus-infected mice

<table>
<thead>
<tr>
<th>Days after Infection</th>
<th>Percentage of Area of Pneumonia (mean ± S.E./CAM)</th>
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<tbody>
<tr>
<td></td>
<td>0 mg/day</td>
</tr>
<tr>
<td>3</td>
<td>36.0 ± 8.5</td>
</tr>
<tr>
<td>5</td>
<td>41.3 ± 8.0</td>
</tr>
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* Significant difference (p < 0.05) vs. control by one-way ANOVA and post hoc Dunnett’s test.

**TABLE 2**

Anti-influenza virus activity and cytotoxicity of CAM in vitro

<table>
<thead>
<tr>
<th>Treatment with CAM</th>
<th>EC_{50} (µg/ml)</th>
<th>CC_{50} (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-treatment</td>
<td>39.3 ± 6.6</td>
<td>44.5 ± 7.7</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>56.4 ± 9.5</td>
<td>55.3 ± 3.8</td>
</tr>
</tbody>
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**Fig. 2.** Effect of CAM on the body weight of influenza virus-infected mice (A), the number of infiltrated cells in the BALF (B), and virus yields in the BALF (C). Mice were intranasally infected with influenza virus, and CAM at 0 ( ), 2 ( ), or 20 ( ) mg/day was orally administered as described in the text. A, ten mice were used in each group. The change of body weight of mice is expressed as the mean ± S.E. in each group (n = 4–10). Dead mice in each group were not included in the weight data. This figure shows a representative result in two independent experiments. *, main effect of CAM at 20 mg/day versus no CAM control by repeated measure two-way ANOVA between days 1 and 3 postinfection, p < 0.01 (group-time interaction, p < 0.0001). B, the number of infiltrated cells in each BALF was determined from four to five mice in each group daily for 1 to 4 days postinfection. Mean number of infiltrate ± S.E. in each group is represented. This figure shows a representative result in three independent experiments. *, main effect of CAM at 20 mg/day versus no CAM control by repeated measure two-way ANOVA between days 2 and 4 postinfection, p < 0.05 (group-time interaction, p = 0.053). C, the virus titers in the BALF were determined from four to five mice in each group daily for 1 to 4 days postinfection. Mean virus yield ± S.E. in each group is represented. This figure shows a representative result in two independent experiments. *, main effect of CAM at 20 mg/day versus no CAM control by repeated measure two-way ANOVA between days 1 and 3 postinfection, p < 0.05 (group-time interaction, p = 0.057).
and NO that form more reactive peroxynitrites in the later stages of infection. The alleviation of pneumonia follows the extensive growth of influenza virus which causes pneumonia. These cells are important lines of defense against influenza infection and make up the major population of infiltrated cells in the BALF of infected mice (Raut et al., 1996). In the lungs of infected mice, $O_2^{-}$ is generated from neutrophils and macrophages (Oda et al., 1998). Thus, neutrophils and macrophages are major cellular sources of the NO and $O_2^{-}$ generation that cause pneumonia. These cells are important lines of defense against influenza infection and make up the major population of infiltrated cells in the BALF of infected mice (Raut et al., 1996). However, an anti-influenza virus assay in vitro demonstrated no direct anti-influenza virus activity of CAM (Table 2). Thus, the reduction of virus yield in CAM-treated mice might be due to the modification of immunological response against influenza infection rather than a direct anti-influenza virus activity of CAM. The protective response activated by CAM at 20 mg/day may result in the experimental efficacy of CAM on influenza infection in mice, reflecting the broad biological activity of macrodilides as immunomodulators (Oda et al., 1994; Suzuki et al., 1997; Sato et al., 1998; Khan et al., 1999).

The number of infiltrated cells in the BALF of CAM-treated mice was significantly lower during the early phase of infection than that seen in untreated mice (Fig. 2B). The development of pneumonia was significantly limited in the late phase of infection (Table 1). The important pathogenic factors in influenza virus-induced pneumonia in mice are $O_2^{-}$ and NO that form more reactive peroxynitrites in the later phase (Akaike et al., 1996). In the lungs of infected mice, $O_2^{-}$ is generated from neutrophils and macrophages (Oda et al., 1989), and NO has been suggested to be produced from alveolar exudate macrophages in the BALF of infected mice (Oda et al., 1989). Thus, neutrophils and macrophages are major cellular sources of the NO and $O_2^{-}$ generation that cause pneumonia. These cells are important lines of defense against influenza infection and make up the major population of infiltrated cells in the BALF of infected mice (Raut et al., 1975; Sweet and Smith, 1980; Peschke et al., 1993). Therefore, the alleviation of pneumonia by CAM in the late phase might result from a reduction of the number of infiltrated cells in the respiratory tract in the early phase. However, CAM has been found to have immunomodulatory activity in vitro (Khan et al., 1999) and reduce virus yields in the BALF of infected mice in the early phase, e.g., at the onset of development of pneumonia (Fig. 2C). IL-12 administration also significantly reduced the virus yield of the respiratory tract in the early phase on day 2 (Fig. 4B). The development of pneumonia follows the extensive growth of influenza virus in the respiratory tract of mice (Akaike et al., 1996). Thus,

Fig. 3. Effect of CAM on the levels of IL-12 (A), IL-10 (B), IL-4 (C), IL-6 (D), and IFN-γ (E) in the BALF of influenza virus-infected mice. CAM at 0 (○), 20 (●), 100 (▲), and 500 (△) mg/kg and BALF was prepared from three mice in each group daily for 1 to 4 days postinfection. Mock-infected mice were orally administered with CAM at 0 (△) or 20 (▲) mg/kg, and BALF was prepared from three mice in each group on day 2 postinfection. The cytokine levels in the BALF were determined by ELISA as described in the text. Vertical bars indicate S.E. in each group. Each figure shows a representative result in two or three independent experiments. Main effect of CAM at 20 mg/day versus no CAM control by repeated measure two-way ANOVA between days 2 and 4 postinfection, $p < 0.05$ (group-time interaction, *$p = 0.063$). **$p < 0.05$ versus no CAM control in infected mice by one-way ANOVA and post hoc Dunnett’s test.

Fig. 4. Effect of intranasal IL-12 administration on the body weight of influenza virus-infected mice (A) and virus yields in the BALF (B). A, influenza virus-infected mice were intranasally administered with IL-12 at 0 (○), 20 (●), 100 (▲), and 500 (△) ng/mouse once on day 2 after infection. The changes of body weights ($n = 3–10$) in a single experiment are expressed as the mean ± S.E. in each group. Dead mice in each group were not included in the weight data. $^*$, main effect of IL-12 at 20 ng/mouse versus no IL-12 control by repeated measure two-way ANOVA between days 2 and 7 postinfection, $p < 0.05$ (group-time interaction, $p = 0.001$). B, infected mice were administered with IL-12 at 0 (○) and 20 (●) ng/mouse once on day 2 after infection, and BALF was prepared from four to five mice in each group daily for 2 to 5 days postinfection. The virus titers in the BALF are expressed as the mean ± S.E. in each group. This figure shows a representative result in two independent experiments. $^*$, main effect of IL-12 at 20 ng/mouse versus no IL-12 control by repeated measure two-way ANOVA between days 2 and 5 postinfection, $p < 0.05$ (group-time interaction, $p = 0.73$).

The administration of chronic respiratory tract infections as an antibiotic agent. Using a murine infection model under specific pathogen-free conditions, histopathological analysis of the lungs of infected mice did not indicate any bacterial contamination (data not shown), indicating that no complication with bacteria occurs in the lungs. The alleviation of pneumonia by CAM was thus not due to the antibacterial activity of CAM.

CAM at 20 mg/day significantly reduced virus yields in the BALF of influenza virus-infected mice on days 1 to 4 postinfection (Fig. 2C). However, an anti-influenza virus assay in vitro demonstrated no direct anti-influenza virus activity of CAM (Table 2). Thus, the reduction of virus yield in CAM-treated mice might be due to the modification of immunological response against influenza infection rather than a direct anti-influenza virus activity of CAM. The protective response activated by CAM at 20 mg/day may result in the experimental efficacy of CAM on influenza infection in mice, reflecting the broad biological activity of macrolides as immunomodulators (Oda et al., 1994; Suzuki et al., 1997; Sato et al., 1998; Khan et al., 1999).

The number of infiltrated cells in the BALF of CAM-treated mice was significantly lower during the early phase of infection than that seen in untreated mice (Fig. 2B). The development of pneumonia was significantly limited in the late phase of infection (Table 1). The important pathogenic factors in influenza virus-induced pneumonia in mice are $O_2^{-}$ and NO that form more reactive peroxynitrites in the later phase (Akaike et al., 1996). In the lungs of infected mice, $O_2^{-}$ is generated from neutrophils and macrophages (Oda et al., 1989), and NO has been suggested to be produced from alveolar exudate macrophages in the BALF of infected mice (Oda et al., 1989). Thus, neutrophils and macrophages are major cellular sources of the NO and $O_2^{-}$ generation that cause pneumonia. These cells are important lines of defense against influenza infection and make up the major population of infiltrated cells in the BALF of infected mice (Raut et al., 1975; Sweet and Smith, 1980; Peschke et al., 1993). Therefore, the alleviation of pneumonia by CAM in the late phase might result from a reduction of the number of infiltrated cells in the respiratory tract in the early phase. However, CAM has been found to have immunomodulatory activity in vitro (Khan et al., 1999) and reduce virus yields in the BALF of infected mice in the early phase, e.g., at the onset of development of pneumonia (Fig. 2C). IL-12 administration also significantly reduced the virus yield of the respiratory tract in the early phase on day 2 (Fig. 4B). The development of pneumonia follows the extensive growth of influenza virus in the respiratory tract of mice (Akaike et al., 1996). Thus,
the reduction of the virus yield and the modification of the nature of infiltrates by CAM in the early phase may be the initial events for reducing infiltration into lungs.

We examined the changes of cytokine levels in the BALF and sera of infected mice to evaluate the early immune response modulated by CAM in influenza infection. CAM did not significantly affect the levels of IL-4, -6, and -10 in the BALF of infected mice (Fig. 3, B–D) and in the sera (data not shown). Without CAM treatment, the levels of IL-4 and -10 were not markedly affected in the BALF and sera on days 1 to 4 after infection in this study. The production of IL-4 and -10 in BALF by influenza infection is not affected (Monteiro et al., 1998) in the mediastinal lymph node of mice (Baumgarth et al., 1994) or in the nasal lavage fluid of human volunteers infected with influenza virus (Fritz et al., 1999). Our observation was consistent with these results. The level of IL-6 increased in the BALF (Fig. 3) and sera (data not shown) after influenza infection, confirming previous observations (Hennet et al., 1992; Peschke et al., 1993; Conn et al., 1995; Fritz et al., 1999). A significant CAM-induced elevation of IL-12 and IFN-γ levels was observed on days 2 and 3, respectively (Fig. 3, A and E). IL-12 is produced in the early phase of influenza infection in mice (Monteiro et al., 1998) from monocytes and macrophages (Fritz et al., 1999). Thus, the source of IL-12 in the lung is probably the activated resident alveolar macrophages and monocytes that are the first lines of defense against influenza infection. Thus, CAM administration to infected mice may be effective in augmenting IL-12 production from such cells. IL-12 is a strong inducer of IFN-γ and important in the development of Th1 cells followed by the generation of cell-mediated immunity (Hsieh et al., 1993; Manetti et al., 1994; Paul and Seder, 1994; Monteiro et al., 1998; Arulanandam et al., 1999). This cytokine also induces cytotoxicity of activated T cells and natural killer cells (Robertson et al., 1992; Kos and Engleman, 1996). Recovery from influenza infection in mice is primarily mediated by anti-influenza CD8+ cytotoxic T lymphocyte (CTL) activity (Eichberger et al., 1991; Bender et al., 1992). In the early phase of influenza infection, IFN-γ and cytotoxic activity may be important factors in leading to the recovery from the infection. Thus, the elevation of IL-12 by CAM may locally promote IFN-γ production leading to the development of an immune response against influenza infection. IL-12 significantly reduced influenza virus yield in the BALF and the loss of body weight of infected mice in the early phase of infection (Fig. 4), consistent with the reduction of virus yield and development of pneumonia in the infected mice treated with CAM, in which the level of IL-12 was locally elevated (Fig. 4A). The intranasal administration of IL-12 was probably effective in switching on a further cascade of cytokines, such as IFN-γ. IL-12 has been shown to contribute primarily to the early development and activation of innate immune response (Bender et al., 1992; Hennet et al., 1992). Thus, the local elevation of IL-12 level could be an important factor contributing to the early development of an anti-influenza virus protective response.

When IL-12 was intranasally administered to influenza virus-infected mice, administration once on day 2 after infection was effective in reducing the loss of body weight of infected mice. In this experiment, an effective dose (20 ng/mouse) was essential in minimizing the loss of body weight (Fig. 4A). Kostense et al. (1998) showed that the intraperito-

neal administration of IL-12 daily for days −1 to 4 after infection delayed recovery from influenza infection in mice. IL-12 production contributing to recovery from influenza infection may be very critical in its level, acting site, and timing after infection. Our results suggest that local and timely, but not systemic, augmentation of IL-12 production can promote the early development and activation of an innate immune response, leading to recovery from influenza. Monteiro et al. (1998) showed that the production of cytokines and CTL activity later in influenza infection was independent of endogenous IL-12 after the neutralization of endogenous IL-12 by anti-IL-12 antibody in the early phase. In our study, significant reduction in the mortality of infected mice following IL-12 administration was not observed, although the mortality was somewhat reduced (data not shown). Thus, in addition to the early immune response, the activation of CTL and the secretion of neutralizing antibody in the late phase of infection would be essential for efficient antiviral immunity to recover from influenza virus infection.

In this study, we have revealed that the augmentation of IL-12 production by CAM or administration of supplementary IL-12 in the respiratory tract was essential in reducing virus yield in the early phase of influenza and might be crucial in leading to recovery from influenza infection. We demonstrated that in infection of influenza in the respiratory tract, IL-12 plays an important role in the early development of a protective response.

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References


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