Immunological analysis and pathological examination of gnotobiotic mice monoassociated with *Mycoplasma pneumoniae*

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Although mycoplasmal pneumonia has been generally considered to be a disease with good prognosis, a pathogenic host immune response has been associated with its occurrence. In the present study, the pathogenic significance of the immune response was examined using germ-free mice either infected intranasally with *Mycoplasma pneumoniae* or inoculated with *M. pneumoniae* antigens (soluble antigen and partially purified antigen). In gnotobiotic mice monoassociated with *M. pneumoniae*, 10⁴ c.f.u. *M. pneumoniae* per lung were isolated 2–28 days after infection. Inflammatory changes with infiltration of lymphocytes were histopathologically detected in the perivascular area at 2 and 7 days after infection. In the mice intranasally inoculated with soluble antigen or partially purified antigens (F6 and F10 antigens), infiltration of neutrophils and lymphocytes was histopathologically detected at 2 days after inoculation. Severe pneumonia with tissue destruction was observed in the mice inoculated with F6 antigen. A gamma interferon (IFN- γ) dominant response in endogenous cytokine expression was observed in all the treated mice. These results indicate that inflammatory changes in the lung tissue were prolonged in gnotobiotic mice monoassociated with *M. pneumoniae* compared with mice inoculated with *M. pneumoniae* antigen. In addition, it was shown that IFN- γ plays an important role in the pathogenesis of pneumonia in mice either infected with *M. pneumoniae* or inoculated with its antigen. In particular, the F6 antigen has been considered to be an important virulence factor in terms of induction of tissue injury causing infiltration of lymphocytes and neutrophils in the lung, suggesting a close interaction between the immune response and the occurrence of *M. pneumoniae* pneumonia.

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INTRODUCTION

Mycoplasma pneumoniae pneumonia is considered to be a relatively benign disease that is improved by appropriate treatment with antimicrobial agents in many cases, even though it presents symptoms such as high fever, persistent cough and dyspnoea. However, in some cases it becomes severe by presenting multivarious clinical features (Shah & Muthiah, 1996), such as the development of bronchial asthma (Yano *et al.*, 1994) and complication by extrapulmonary lesions (Fernald *et al.*, 1975).

The presence of potential pathogenic factors, such as extracellular membrane elements, including LPS, is not detected in *Mycoplasma* species unlike other pathogenic bacteria. The impairment of the airway membrane by oxyradicals has been reported to be a pathogenic factor of *Mycoplasma pneumoniae* (Yano *et al.*, 1994; Fernald *et al.*, 1975; Chiou *et al.*, 1997; Izumikawa *et al.*, 1986). However, as the pathogenicity of *M. pneumoniae* is not so potent, it is difficult to explain the mechanism by which pneumonia and its complications develop merely through direct cell impairment with this bacterium. It has been reported that a delayed type allergic reaction results from intracutaneous reaction due to *M. pneumoniae* antigen (Mizutani *et al.*, 1971). Association of cellular immunity with the

Abbreviations: FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IFN- γ , gamma interferon; IL, interleukin; PE, phycoerythrin.

development of mycoplasmal pneumonia has been implicated (Denny *et al.*, 1972). From such background knowledge, an indirect mechanism via the host immune response is considered to be more important than direct impairment (Fernald & Clyde, 1976; Fernald *et al.*, 1981).

Hamsters (Fernald *et al.*, 1972) and guinea pigs (Brunner, 1981) have been used as models to analyse pathogenic factors in mycoplasmal pneumonia. In these animals, however, detailed experimental results have not been obtained as not enough reagents for immunological analysis are available. On the other hand, reagents for immunological analysis of mice can be easily obtained, but *M. pneumoniae* infection in mice rarely develops into pneumonia. Given this situation, *Mycoplasma pulmonis* has been used in place of *M. pneumoniae*. Differences in the histopathological patterns of pneumonia induced by *M. pulmonis* between mice with different genetic backgrounds have been reported (Brunner, 1997). However, *M. pulmonis* pneumonia does not necessarily reflect pneumonia caused by *M. pneumoniae*, a human pathogen.

We have previously reported that pneumonia can be caused by intranasal inoculation of germ-free mice with *M. pneumoniae* (Hayakawa *et al.*, 2002). In the present study, we prepared gnotobiotic mice monoassociated with *M. pneumoniae*, which enabled us to analyse the intrinsic pathogenicity of *M. pneumoniae* and host immune reactions without being influenced by other micro-organisms, and studied the role of lymphocytes and cytokines in *M. pneumoniae* infection. Furthermore, we intranasally inoculated germ-free mice with *M. pneumoniae* soluble antigen or partially purified antigens, and made a time-course comparison of the histopathology of the lung and the antigen-specific gamma interferon (IFN- γ) and interleukin 4 (IL-4) induction activity of pulmonary lymph nodes and splenic lymph nodes in order to elucidate the mechanism by which pneumonia develops.

METHODS

Bacterial strains. The M129 strain of *M. pneumoniae* was used in the experiments, and was obtained from the Department of Infectious Diseases, Kyorin University School of Medicine. Cultivation of *M. pneumoniae* was carried out at 37 °C for 7 to 10 days under an atmosphere of 5% CO₂ using PPLO broth (Oxoid) with *Mycoplasma* selective supplement-G (M-suppl) (Oxoid).

Germ-free mice and breeding conditions. Germ-free mice (IQI/Jic, 8-week-old females) were purchased from Clea Japan and bred in vinyl isolators (Clea Japan), which were sterilized by Expur (Alcide). The mice were fed a ⁶⁰Co-irradiated diet (Clea Japan) and sterilized water. Prior to the experimental infection, asepsis was confirmed using control mice (*n*=2) by aerobic and anaerobic culture of the caecum contents on Columbia agar supplemented with 5% sheep's blood. In addition, lung tissue was homogenized (*n*=2 control mice), and was inoculated onto PPLO agar plates with M-suppl, and cultured at 37 °C for 7 to 10 days under an atmosphere of 5% CO₂, to confirm that there was no *M. pneumoniae* infection in the breeding colony.

Preparation of *M. pneumoniae* soluble antigen. A 100 ml culture of *M. pneumoniae* (6×10^7 c.f.u. ml⁻¹) was centrifuged at 3000 *g* for 25 min, and the pellets were resuspended in 10 ml Hanks' balanced salt solution (Invitrogen), and then washed twice with further centrifugation at 3000 *g* for 20 min. Soluble antigen was obtained by suspending the pellets in 5 ml sterilized water and sonicating ten times for 1 min with a Sonifier 250 (Branson Ultrasonics). After centrifugation at 3000 *g* for 5 min, the protein concentration of the supernatant was measured using the Bio-Rad protein assay at 595 nm absorbance and stored at -80 °C until use.

Preparation of *M. pneumoniae* partially purified antigens. *M. pneumoniae* soluble antigen was partially purified by gel filtration with Superdex 200 (Pharmacia). Column chromatography was performed using buffer (50 mM sodium phosphate, 0.15 M NaCl, pH 7.0) at a flow rate of 1.0 ml min⁻¹ with a Pharmacia FPLC system. *M. pneumoniae* soluble antigen (1 ml) was applied to the column (capacity 50 ml), and then partially purified antigen was collected with a fraction collector (Pharmacia LKB FRAC-100). Partially purified antigens were concentrated twofold at 3000 *g* for 20 min with a Centriplus centrifugal filter device (Millipore).

Activity of *M. pneumoniae* partially purified antigens. MOLT-4 cells (human leukaemia cell line) (Dainihon Seiyaku) were adjusted to 1×10^6 cells ml⁻¹ in RPMI 1640 (Invitrogen) with 5% fetal bovine serum (FBS; Invitrogen). Then 2 μ g *M. pneumoniae* partially purified antigens and 2 μ g gentamicin (Schering-Plough) was added to each well. MOLT-4 cells were incubated at 37 °C under an atmosphere of 5% CO₂ for 72 h. Supernatants from these cultures were collected by centrifugation at 700 *g* for 5 min and stored at -80 °C until use. Quantitative analysis of IFN- γ and IL-4 in the supernatants was carried out by ELISA. The following cytokine kits were used in this study: mouse IFN- γ anti-human ELISA and mouse IL-4 anti-human ELISA (BioSource).

Inoculation of germ-free mice with *M. pneumoniae* and its antigens (soluble and partially purified). The experimental group was composed of five mouse groups: those infected with *M. pneumoniae* (*n*=8), those inoculated with *M. pneumoniae* soluble antigen (*n*=4), partially purified antigen fraction 6 (*n*=4) and fraction 10 (*n*=4) and those inoculated with normal saline as a control group (non-infected mice) (*n*=4). IQI/Jic germ-free mice (8 weeks old) were inoculated intranasally under anaesthesia by intraperitoneal injection of pentobarbital (Dainihon Seiyaku). The inoculum (30 μ l) of the M129 strain for the first infection was 1.8×10^5 c.f.u. per mouse. The inoculum (30 μ l) of *M. pneumoniae* soluble antigen and partially purified antigens for the first inoculation was 5 μ g per mouse. The second inoculation was carried out 4 weeks after the primary inoculation by the same method. A total of 30 μ l of saline was similarly given to the control mice.

Bacteriological examination of the infected mice. A total of 3 ml RPMI 1640 was added to resected lung, which was emulsified using a sterilized glass homogenizer (Ikemotorika Kogyo). The emulsified specimen was serially diluted in PPLO broth and inoculated onto PPLO agar plates at 37 °C under an atmosphere of 5% CO₂. The number of *M. pneumoniae* c.f.u. counts in the lung was estimated from the number of colonies formed. *M. pneumoniae* colonies showing typical nipple-like morphology were observed by an inverted microscope.

Pathological examination of infected and inoculated mice. Lungs excised from mice were expanded with pressure from a syringe loaded with 10% formalin solution. After fixation of the samples they were dehydrated, and after embedding in paraffin slices were made, which were stained with haematoxylin and eosin, Elastica Masson and Elastica van Gieson stains.

Immunological examination of infected and inoculated mice

Detection of *M. pneumoniae* antibody. Antibody titres to *M. pneumoniae* in the serum of *M. pneumoniae*-infected mice and -inoculated mice were measured by the particle agglutination test, using a Serodia MycoII kit (Fujirebio), in order to confirm whether the immune response to *M. pneumoniae* was induced. Specimens showing agglutination of sensitized particles (1:40 final dilution) were interpreted as positive.

Detection of lymphocyte subsets in the lung and spleen. To evaluate the intrapulmonary immune response, the lymphocyte subsets in the lungs were analysed. Lungs were removed and minced in 5 ml RPMI 1640 with 5 % FBS containing 50 U collagenase type I ml⁻¹ (Worthington Biochemical). Then the solution was incubated at 37 °C for 30 min and passed through a nylon mesh (Becton Dickinson). The cell suspension was pelleted by centrifugation at 300 g for 10 min, followed by washing three times in RPMI 1640 with 5 % FBS. The cells were overlaid on Lympholyte-M (density=1.0875 g cm⁻³) (Cedarlane) and centrifuged at 500 g for 20 min for separation of lymphocytes.

For fluorescence-activated cell sorting analysis (Hussell *et al.*, 1996), the lymphocytes were washed and adjusted to 2×10^6 cells ml⁻¹ in PBS without calcium chloride and magnesium chloride [PBS (-)]. Aliquots (500 µl) of the cell suspensions were incubated with fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated mAbs to various CD antigens described below for 60 min in PBS. The labelled cells were analysed by flow cytometry (FACS Vantage; Becton Dickinson). The following mAbs were used in this study: rat anti-mouse IFN- γ (FITC labelled) (Becton Dickinson) and rat anti-mouse IL-4 (PE labelled) (Becton Dickinson).

To evaluate the systemic immune response, the lymphocyte subsets in the spleens were examined. The spleens were minced in RPMI 1640 with 5 % FBS and passed together through a nylon mesh. The lymphocytes were obtained and analysed by flow cytometry. The following mAbs were used in this study: rat anti-mouse IFN- γ (FITC labelled) (Becton Dickinson) and rat anti-mouse IL-4 (PE labelled) (Becton Dickinson).

Cytokine productivity of lung and spleen cells. Cytokine productivity of the spleen and lung lymphocytes of the infected mice was examined. The lymphocytes of the spleen and lung were

adjusted to 10^6 cells ml⁻¹ in RPMI 1640 with 5 % FBS and seeded in 200 µl complete RPMI 1640 with 5 % FBS in flat-bottomed microtitre plates (Iwaki), followed by incubation at 37 °C for 24 h under an atmosphere of 5 % CO₂. Then 10 µl *M. pneumoniae* culture (1.2×10^4 c.f.u. per well), *M. pneumoniae* soluble antigen (2 µg per well) or *M. pneumoniae* partially purified antigens (2 µg per well) and gentamicin (2 µg per well) were added to each well. The lymphocytes were incubated at 37 °C under an atmosphere of 5 % CO₂ for 72 h. Supernatants from these cultures were collected by centrifugation at 300 g for 10 min and stored at -80 °C until use.

Quantitative analysis of IFN- γ and IL-4 in the supernatants was carried out by ELISA. The following cytokine kits were used in this study: mouse IFN- γ anti-mouse ELISA (BioSource) and mouse IL-4 anti-mouse ELISA (BioSource).

RESULTS

Gel filtration chromatography of *M. pneumoniae* soluble antigen

M. pneumoniae soluble antigen was applied to gel filtration columns, and IFN- γ and IL-4 inducing activities in the fractions separated were examined (Fig. 1). With the addition of fraction 6 (F6) to cells, the secretion of IFN- γ (92.2 pg ml⁻¹) and IL-4 (62.4 pg ml⁻¹) was detected in the supernatant solution. Likewise, IFN- γ (78.8 pg ml⁻¹) and IL-4 (45.7 pg ml⁻¹) were detected with the addition of fraction 10 (F10) to cells. The induction of cytokine production by other partially purified fractions was not found.

Number of bacteria in mouse lung after nasal *M. pneumoniae* infection

Time-course changes in the number of bacteria in the lung of gnotobiotic mice mono-associated with *M. pneumoniae* were assessed, and neither a significant increase nor a decrease in the number of bacteria was found throughout

Fig. 1. Purification of antigen from *M. pneumoniae* soluble antigen by FPLC using Superdex 200. Each fraction was used for ELISA to evaluate induction activity of IL-4 and IFN- γ secretion from MOLT-4 cells.

the time-course. *M. pneumoniae* were recovered at 1.7×10^4 , 4.4×10^4 , 4.5×10^4 and 1.9×10^4 c.f.u. per lung at 2, 7, 14 and 28 days post-infection (repeated infection), respectively.

Changes in mouse serum antibody titre after nasal inoculation with *M. pneumoniae* or nasal administration with *M. pneumoniae* antigen

Changes in the anti-*M. pneumoniae* antibody titre are shown in Fig. 2. The antibody titre in the *M. pneumoniae* infection group increased fourfold to eightfold during the period from 2 to 28 days after infection. The antibody titre increased from 8-fold to 24-fold during the period from 2 to 7 days after inoculation in the soluble antigen inoculation group. The antibody titre showed a 4-fold to 48-fold increase in the F6 inoculation group and a 4-fold to 32-fold increase in the F10 inoculation group.

Histological findings

***M. pneumoniae* infection group.** Histological findings in the non-infected control group are shown in Fig. 3(a). In the *M. pneumoniae* infection group, 2 days after infection pulmonary tissues showed infiltration mainly of lymphocytes in the interstice around pulmonary blood vessels, as well as mild infiltration of lymphocytes in the alveolar septum and thickening of the alveolar wall (Fig. 3b). These findings persisted until 7 days after infection (Fig. 3c), decreased 14 days after infection (Fig. 3d), and histological findings similar to those of the non-infected mouse were found 28 days after infection (Fig. 3e).

***M. pneumoniae* soluble antigen inoculated mouse group.** Pulmonary tissues 2 days after inoculation of the soluble antigen showed mainly infiltration of lymphocytes

in the interstice around pulmonary blood vessels and partial infiltration of neutrophils, infiltration of these cells in the surrounding alveolar septum and the alveolus, and thickening of the alveolar wall attendant upon lymphocyte infiltration (Fig. 4a). These findings decreased to the extent of only lymphocytes being found 7 days after inoculation (Fig. 4b).

***M. pneumoniae* partially purified antigen F6 inoculated mouse group.** Pulmonary tissues 2 days after partially purified antigen F6 inoculation showed a marked inflammatory cell infiltration, mainly with monocytes that extended from the periphery of pulmonary blood vessels to the bronchus and bronchovesicular region, in addition to a localized intense inflammatory pattern with accumulation of neutrophils, nuclear destruction, detached alveolar tissues and effusion (Fig. 4c). In these regions of intense inflammatory cell infiltration, alveolar lumen full of segmented cells, large polykaryocytes and nuclear destruction was observed by Elastica Masson staining (Fig. 5a, b). Furthermore, the loss of irregular basement membrane, destruction of the alveolar structure, and intracellular nuclear breakage were found by Elastica van Gieson staining (Fig. 5c, d). Seven days after the inoculation, the infiltration of these inflammatory cells improved to the extent of mild lymphocyte infiltration being found in the interstice around the pulmonary blood vessels and the alveolar septum (Fig. 4d).

***M. pneumoniae* partially purified antigen F10 inoculated mouse group.** Infiltration mainly of lymphocytes was found in the region around the bronchial wall, and interstice around pulmonary blood vessels and alveolar septum 2 days after F10 inoculation (Fig. 4e). Seven days thereafter, the infiltration improved to the extent of only a small number of lymphocytes being found in the interstice around pulmonary blood vessels (Fig. 4f).

Fig. 2. Anti-*M. pneumoniae* antibody titre measured by particle agglutination. Each bar indicates the mean of the anti-*M. pneumoniae* antibody titre ($n=2$). White bars, *M. pneumoniae*; black bars, sonicate; hatched bars, F6; grey bars, F10. *, Antibody titre was not measured in the germ-free mice treated with the soluble antigen, or the partially purified antigens F6 and F10.

Intrinsic and secretory cytokines in pulmonary and splenic lymphocytes of *M. pneumoniae* M129 infected gnotobiotic mice

The intrinsic IFN- γ -positive rate in pulmonary lymphocytes isolated from mice 2 and 7 days after inoculation with the *M. pneumoniae* M129 strain was high at 2.25 and 2.32 %, respectively, compared with 0.07 % in the *M. pneumoniae* non-infected mice (Fig. 6a). However, this positive cell rate decreased 14 and 28 days after infection. In splenic lymphocytes too, the intrinsic IFN- γ -positive cell rate 2 and 7 days after infection was high at 4.6 and 3.01 %, respectively, compared with 0.18 % in non-infected lymphocytes, but it decreased 7 and 14 days after infection. On the other hand, the intrinsic IL-4-positive cell rate in pulmonary lymphocytes was low 7 and 14 days after infection, while the intrinsic IL-4-positive cell rate in splenic lymphocytes increased slightly to 1.0 % 7 days after infection.

Fig. 3. Histopathological findings for the lung tissue. Lung was fixed and stained with haematoxylin and eosin after intranasal infection of mice with *M. pneumoniae* strain M129 (magnification $\times 200$). (a) Uninfected control, (b) 2 days post-infection, (c) 7 days post-infection, (d) 14 days post-infection and (e) 28 days post-infection.

Secretory IFN- γ induced from pulmonary lymphocytes was not detected in *M. pneumoniae* non-infected mice, but it showed a slightly high level at 13.6 pg ml^{-1} 7 days after infection, while secretory IFN- γ induced from splenic lymphocytes remained low throughout the time-course (Fig. 6b). Secretory IL-4 from pulmonary lymphocytes was not detected, and secretory IL-4 from splenic lymphocytes was also low at 1.0 pg ml^{-1} .

Intrinsic and secretory cytokines in pulmonary and splenic lymphocytes of *M. pneumoniae* soluble antigen inoculated mice

The intrinsic IFN- γ -positive cell rate in soluble antigen inoculated mice was high at 1.55 and 3.12% 2 and 7 days after inoculation in pulmonary lymphocytes, respectively, compared with 0.85% in the non-inoculated mice (Fig. 7a). Likewise in splenic lymphocytes, it was high at 2.80 and 3.52% 2 and 7 days after inoculation, respectively, compared with the non-inoculated group. However, the intrinsic IL-4-positive cell rate was low in both pulmonary and splenic lymphocytes. Secretory IFN- γ in pulmonary lymphocytes was not detected in non-inoculated mice, but it showed high levels at 125.6 pg ml^{-1} and 214 pg ml^{-1} 2 and 7 days after inoculation of soluble antigens (Fig. 7b), respectively. Secretory IFN- γ in splenic lymphocytes showed high levels at 97.2 pg ml^{-1} and 166.9 pg ml^{-1} 2 and 7 days after inoculation of soluble antigens, respectively, compared with

42.5 pg ml^{-1} in non-inoculated mice. However, secretory IL-4 was low in both pulmonary and splenic lymphocytes.

Intrinsic and secretory cytokines in pulmonary and splenic lymphocytes of *M. pneumoniae* partially purified antigen F6 inoculated mice

The intrinsic IFN- γ -positive cell rate in *M. pneumoniae* partially purified antigen F6 inoculated mice was high compared with non-inoculated mice 2 days after inoculation in pulmonary and splenic lymphocytes (Fig. 7c). However, the intrinsic IL-4 positive cell rate was low in both pulmonary and splenic lymphocytes. The secretory IFN- γ rate was low in both pulmonary and splenic lymphocytes (Fig. 7d). Secretory IL-4 was 8.1 pg ml^{-1} 2 days after inoculation in pulmonary lymphocytes and 640 pg ml^{-1} 7 days after inoculation in splenic lymphocytes.

Intrinsic and secretory cytokines in pulmonary and splenic lymphocytes of *M. pneumoniae* partially purified antigen F10 inoculated mice

The intrinsic IFN- γ -positive cell rate was high at 11.9 and 2.24% 2 and 7 days after inoculation, respectively, in pulmonary lymphocytes compared with the non-inoculated groups (Fig. 7e). In splenic lymphocytes, it showed a high level (3.58%) 7 days after inoculation. However, the intrinsic IL-4-positive cell rate showed low levels of less

Fig. 4. Histopathological findings for the lung tissue of the germ-free mice inoculated with *M. pneumoniae* soluble antigens (a, b), and the partially purified antigens F6 (c, d) and F10 (e, f). The lung was fixed and stained with haematoxylin and eosin after intranasal inoculation (magnification $\times 200$). (a, c, e) 2 days post-inoculation; (b, d, f) 7 days post-inoculation.

than 1% 2 and 7 days after inoculation in both pulmonary and splenic lymphocytes. Secretory IFN- γ showed low levels in pulmonary lymphocytes (Fig. 7f). Secretory IFN- γ in splenic lymphocytes was not high at 18.5 and 16.5 pg ml $^{-1}$ 2 and 7 days after inoculation. However, secretory IL-4 in pulmonary and splenic lymphocytes showed low levels.

DISCUSSION

Mycoplasmal pneumonia has a relatively good prognosis, but it sometimes shows pathological features such as extrapulmonary complications and exacerbation of pneumonia. In *M. pneumoniae*, the toxicity of the bacterium itself is weak. An indirect lesion induced by *M. pneumoniae*

Fig. 5. Histopathological findings for the lung tissue of germ-free mice inoculated with partially purified antigen F6. The lung was fixed and stained with Elastica Masson and Elastica van Gieson stain 2 days after intranasal inoculation with the partially purified antigen F6 of *M. pneumoniae* strain M129. (a) Elastica Masson stain (magnification $\times 200$), (b) Elastica Masson stain (magnification $\times 400$), (c) Elastica van Gieson stain (magnification $\times 200$), (d) Elastica van Gieson stain (magnification $\times 400$).

Fig. 6. Positive percentages of intrinsic cytokines in lymphocytes (a) and production of cytokines from lymphocytes (b) recovered from the lung and the spleen of gnotobiotic mice infected with *M. pneumoniae* strain M129. N, Non-infected; *, below the detectable limit.

is considered to be important and constitute a main element of such pathology (Atkinson *et al.*, 2008). It has been reported that there is infiltration of inflammatory cells into the alveolar lumen 3 days after intranasal infection with *M. pulmonis* and into the interstice around pulmonary blood vessels 7 days after intranasal infection with *M. pulmonis* (Tanaka *et al.*, 1986).

However, the pneumonia pattern becomes conspicuous as *M. pneumoniae* infection is induced in animal experiments (Fernald *et al.*, 1981). We have made it clear from experiments conducted using germ-free mice infected with *M. pneumoniae* that the lungs do not present inflammatory patterns after the first infection, but that pneumonia is caused 14 days after reinfection (Hayakawa *et al.*, 2002). We showed that an intense inflammation is induced 2 and 7 days after *M. pneumoniae* reinfection and that pneumonia disappears 14 and 28 days after the reinfection. Such a marked pneumonia pattern after reinfection, together with the fact that the incidence of human mycoplasmal pneumonia is scarce in infancy and increases after school age, suggests that the immune response due to repeated exposure to *M. pneumoniae* antigens is closely connected with pulmonary pathology.

Naot *et al.* (1981) have reported that a similar inflammatory pattern as found for *M. pulmonis* infection is found histologically when LEW rats are intranasally inoculated with killed *M. pulmonis*. It was reported that intrabronchial inoculation of C57BL/6j mice and BALB/c mice with *M. pneumoniae* bacterial content causes pneumonia (Watanabe, 1999). In conventional mice, however, the involvement of other bacteria cannot be ruled out since the animals are always in contact with the external environment. In reports by Takahashi *et al.* (2000) and Taguchi *et al.* (2002), germ-free mice were used as an experimental model for intestinal tract infections. Germ-free mice lacking intestinal flora enable us to observe the monoassociated interaction between the pathogen and the host. By using germ-free mice, therefore, we were able to observe a pure immune response free of the influence of other bacteria.

Having no cell wall, *M. pneumoniae* is supposed to have no marked virulence factor such as LPS. However, it has been reported that the membrane component lipoprotein of *Mycoplasma salivarium* and *Mycoplasma fermentans* (Kataoka *et al.*, 2006) induce tumour necrosis factor alpha from lymphocytes and cause apoptosis, suggesting that the bacterial membrane component of mycoplasma contains an immune system stimulatory substance. Recently, it has been reported that *M. pneumoniae*-derived lipopeptides induced acute inflammatory responses in the lung of mouse (Shimizu *et al.*, 2008).

We identified active *M. pneumoniae* bacterial components in the present study; partially purified antigen F6 led to accumulation of lymphocytes and neutrophils in the lung and caused pneumonia. The specific substances derived from *M. pneumoniae* that caused the immune response had not been discovered, so we intended to evaluate a new pathogenic factor by carrying out purification by FPLC. However, high purification and identifying the amino acid sequence of F6, and the specifying encoding gene, remain to be determined.

It has been reported that *M. pulmonis* has mitogenic activity against lymphocytes (Naot & Ginsburg, 1978). Pietsch & Jacobs (1993) have reported that mRNA expression of tumour necrosis factor alpha, IFN- γ and IL-6 of pulmonary and splenic lymphocytes was observed more intensively when BALB/c mice were repeatedly infected with *M. pneumoniae* than with a single infection. When germ-free mice were reinfected with *M. pneumoniae* in the present study, inflammatory patterns were seen 2 and 7 days after infection. IFN- γ in the lung showed high levels and a Th1-dominant immune reaction was found. Likewise, an IFN- γ -dominant induction was seen with respect to soluble antigen, and partially purified antigens F6 and F10. In the present study, inflammatory patterns and an increase in the production of pulmonary lymphocyte IFN- γ were found, and a time-course decrease of the inflammatory changes in pulmonary tissues with a decline

Fig. 7. Positive percentages of intrinsic cytokines in lymphocytes (a, c, e) and production of cytokines from lymphocytes (b, d, f) recovered from the lung and the spleen of germ-free mice. Mice were inoculated with *M. pneumoniae* soluble antigen (a, b), partially purified antigen F6 (c, d) and partially purified antigen F10 (e, f). N, Non-infected; *, below the detectable limit.

of IFN- γ was seen particularly in the F6 and F10 inoculation groups. Such Th1 cytokine inducing changes are considered to be similar in the clinical course from the development to cure of mycoplasmal pneumonia. Murine studies also have shown that *M. pneumoniae* infection induces a T helper 1 cytokine response in the lung (Hardy *et al.*, 2001; Fonseca-Aten *et al.*, 2005). Since splenic lymphocytes have cytokine secreting activity, *M. pneumoniae* infection is likely to cause an immune reaction not only in the lung but also in other organs. It is possible that the immune reaction plays an important role in forming

extrapulmonary lesions in mycoplasmal infection (Atkinson *et al.*, 2008). Yano *et al.* (1994) have reported that IgE antibody is present in serum mycoplasmal pneumonia patients in particular and that the hypersensitivity reaction in the pulmonary tract increases with inoculation of *M. pneumoniae* antigen. However, it has been reported that an *M. pneumoniae* gene is detected at high percentages by PCR from pharyngeal smears of patients for whom symptoms of bronchial asthma are stabilized (Kraft *et al.*, 2002), suggesting the possibility that the settlement of bacteria and continuous immune

reactions in mycoplasmal infections may explain the clinical feature that coughing is prolonged even when fever, inflammatory reaction data and chest X-ray patterns have been improved.

The present experiments have made it clear that inflammation can be produced in the lungs of germ-free mice inoculated with *M. pneumoniae* and bacterial components of *M. pneumoniae*. As a result of the time-course observations after reinoculation, intense reactions in the early stage, and inflammation caused by live bacteria and components of bacterial cells, suggest that the development of mycoplasmal pneumonia is closely related to the immune reaction.

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