change of temperature is critical for helicobacter survival in the river environment (Shahamat *et al.* 1993; Adans *et al.* 2003; Azevedo *et al.* 2004, 2008).

As shown in several *in vitro* studies, which indicate that acanthamoeba can tolerate survival and multiplication of human pathogens (Greub and Raoult 2004), most of our current knowledge of microbial interactions in the natural environment has been obtained from simplified laboratory experiments with micro-organisms taken out of the field. However, natural environments, such as river water and soil, contain a diverse population of micro-organisms and are extremely complex, and it is not easy to recreate the microcosm in the test tube. Our results suggest that a possible role of acanthamoeba as an environmental reservoir of helicobacter may be limited as both are coincidentally present in the environment. However, further studies, including a more extensive survey, development of PCR assays that are more highly specific for detection of helicobacter, and *in vitro* experiments that accurately reflect actual field conditions need to progress to determine whether or not acanthamoeba is a natural reservoir for helicobacter including *H. pylori* in the natural environment.

Acknowledgements

Kawaguchi K and Matsuo J contributed equally to this work. This study was supported by the Waksman foundation of Japan, the Asahi Glass foundation of Japan, and the Ohyama Health foundation of Japan.

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Journal of Medical Microbiology (2009), 58, 656–662 DOI 10.1099/jmm.0.008185-0

Vertical *Helicobacter pylori* transmission from Mongolian gerbil mothers to pups

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To identify the time frame and route of mother-to-child *Helicobacter pylori* infection, a Mongolian gerbil model was used. Four-week-old female Mongolian gerbils were infected with *H. pylori*, and then mated with uninfected males 2 months after infection. The offspring were sacrificed weekly after birth, and then serum, mother's milk from the stomach and gastric tissues were obtained from pups. Anti-*H. pylori* antibody titres were measured in sera and maternal milk using an ELISA. The stomach was cut in two in the sagittal plane, and then *H. pylori* colonization in mucosa was confirmed by culture and real-time RT-PCR in one specimen and by immunochemical staining in the other. Faeces and oral swabs were obtained from infected mothers, and *H. pylori* 16S rRNA was measured using real-time RT-PCR. *H. pylori* was not identified in cultures from the gastric mucosa of pups delivered by infected mothers, but *H. pylori* 16S rRNA was detected from 4 weeks after birth, suggesting that Mongolian gerbil pups become infected via maternal *H. pylori* transmission from 4 weeks of age. The anti-*H. pylori* antibody titre in sera of pups from infected mothers was maximum at 3 weeks of age and then rapidly decreased from 4 weeks of age. High antibody titres in mother's milk were detected during the suckling period, and GlcNAcα was detectable at 2–4 weeks of age, but disappeared as the offspring aged. Thus *H. pylori* seems to infect Mongolian gerbil pups from 4 weeks of age, in parallel with decreasing GlcNAcα expression in the gastric mucosa. These results suggested that *H. pylori* infection of Mongolian gerbil pups occurs via faecal–oral transmission from an infected mother.

Received 21 November 2008
Accepted 15 January 2009

INTRODUCTION

Helicobacter pylori, a spiral-shaped pathogenic bacterium found in human gastric mucosa, was originally isolated by Warren and Marshall (Warren & Marshall, 1983) in 1982 and it was soon linked with chronic antral gastritis and peptic ulceration (Marshall & Warren, 1984).

Infection with *H. pylori* can persist for years or for a lifetime (Rowland, 2000; Taylor *et al.*, 1995), although spontaneous clearance often occurs in childhood (Granstrom *et al.*, 1997; Malaty *et al.*, 1999). Infection with *H. pylori* is closely associated with gastritis and peptic ulcers (Goodwin, 1997; NIH Consensus Conference, 1994), and it is a risk factor for gastric cancer (IARC Working Group, 1994) and mucosa-associated lymphoid tissue lymphoma (Blecker *et al.*, 1995). The mode(s) of *H. pylori* transmission is not fully understood despite considerable research. Epidemiological studies have demonstrated the importance of close person-to-person contact and intrafamilial spread (Drumm *et al.*, 1990), but whether this bacterium is primarily transmitted through the faecal–oral or oral–gastric route remains uncertain (Parsonnet *et al.*, 1999). Others (Kivi *et al.*, 2003; Konno *et al.*, 2005; Malaty *et al.*, 1991; Oderda *et al.*, 1991) have suggested intrafamilial clustering of *H. pylori* infections, and molecular DNA analyses of familial *H. pylori* strains have indeed revealed intrafamilial infections with a single *H. pylori* strain (or a common source of infection within the family) (Bamford *et al.*, 1993; Nwokolo *et al.*, 1992). Transmission among family members is considered to constitute the main route of *H. pylori* infection (Covacci *et al.*, 1999). Infected parents, particularly mothers, have been considered likely to play a key role in the intrafamilial transmission of *H. pylori* (Rothenbacher *et al.*, 1999; Brenner *et al.*, 2000; Weyermann *et al.*, 2006), whereas environmental factors might be more important than intrafamilial transmission in developing countries (Rothenbacher *et al.*, 2000; Sarker *et al.*, 1997). However, these epidemiological studies were not based on molecular biological analyses of *H. pylori* strains.

Here, we used molecular analyses to examine *H. pylori* transmission in the stomachs of Mongolian gerbil pups, as well as in the faeces and oral cavity of infected or

non-infected mothers. We also examined how and when Mongolian gerbil pups become infected with *H. pylori* derived from their mothers.

METHODS

Animals. Four-week-old Mongolian gerbils (MGS/Sea; specific pathogen free; body weight 20–30 g) purchased from Kyudo were maintained in plastic cages under standard laboratory conditions (room temperature 23±2 °C; relative humidity 40–60 %; 12 h light–dark cycle) and fed with a standard diet (CE-2; Clea Japan) and sterilized tap water *ad libitum*.

Bacterial strain. We inoculated Mongolian gerbils with *H. pylori* TK1402, which was isolated from gastric biopsy specimens of a patient with gastric and duodenal ulcers. Analysis of the strain using PCR indicated the presence of the vacuolating cytotoxin gene (*vacA*) and the *cagA* gene. We used the primer set F1 and B1 to detect *cagA*, and the primer sets VA3-F and VA3-R and VA4-F and VA4-R to detect *vacA* (Atherton *et al.*, 1995). Genomic DNA (1 µl) extracted using MagExtractor (Toyobo) was mixed with each primer (5 pmol) and 0.5 U *Taq* polymerase in a final volume of 20±1 µl. The PCRs proceeded for 30 cycles at 94 °C for 1 min, 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min using a thermal cycler (GeneAmp PCR system 9600-R; Perkin-Elmer). The bacterial strain expressed Lewis^y antigen (Le^y) on the cell surface. The bacteria were cultured for 2 days in Brucella broth containing 1.5 % agar (Difco) and 7 % horse serum (Shiguma) (BHS-agar) under microaerobic conditions at 37 °C using GasPak jars (Mitsubishi Gas Chemical) containing 85 % N_2, 10 % CO_2 and 5 % O_2.

Preparation of *H. pylori* and inoculation. *H. pylori* was incubated on BHS-agar at 37 °C for 48 h under a microaerophilic atmosphere, suspended to a final density of 1.0×10^9 c.f.u. per ml Hanks' balanced salt solution (HBSS) and then orally administered to Mongolian gerbils twice at a 1 day interval. The sera of challenged animals were confirmed as being *H. pylori*-antibody positive by ELISA as described by Osaki *et al.* (2006). Anti-*H. pylori* antibody was detected in all of the gerbils inoculated.

Experimental design. Two months after infection with *H. pylori*, infected female Mongolian gerbils and uninfected males were transferred to separate cages for mating. As soon as pregnancy was confirmed, infected females were separated from the group and cared for until delivery, when mother and pups were housed in one cage per family. Two to four litters were sacrificed under anaesthesia with diethyl ether at 0, 1, 2, 3, 4, 5, 6, 7, 10 and 22 weeks post-partum. Numbers of micro-organisms in the stomach, and antibody titres against *H. pylori* in serum and ingested milk, were then determined as described below. One or two control uninfected gerbils were sacrificed at the same time as the infected animals.

Fresh faeces and throat swabs obtained from infected and uninfected mothers at 2 and 4 months after *H. pylori* inoculation were examined by real-time RT-PCR as described below.

The experiments were approved by the Experimental Animal Ethics Committee at Kyorin University School of Medicine. All experimental animals were housed in the specific-pathogen-free unit of the animal facility and provided with sterile bedding, food and water.

Quantification of gastric *H. pylori in vitro*. Stomachs extirpated from sacrificed gerbils at various times were dissected along the greater curvature and the contents were removed. The stomachs were then divided longitudinally into two halves, including the fore-stomach-to-pylorus region. The mucous layer collected from the stomach using Spartel was homogenized in 1 ml HBSS and then the homogenate was inoculated onto *H. pylori* selective medium (Nissui Pharmaceutical) and incubated at 37 °C for 5 days. Thereafter, purple colonies were counted and the numbers of viable *H. pylori* in the gastric mucosa were calculated.

Real-time RT-PCR assay. Real-time RT-PCR proceeded as described by Osaki *et al.* (2006). Tissue samples were lysed by vortex mixing with lysozyme (400 µg ml^{-1}) in 100 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA; pH 8) and then mixed with lysis buffer containing guanidine isothiocyanate. Total RNA isolated from the lysate according to the instructions provided with the RNeasy Mini kit (Qiagen) was quantified by measuring the A_{260}/A_{280} ratio. Contaminating chromosomal DNA was digested with DNA-free DNase I [1 U (µg RNA)$^{-1}$; Ambion] at 37 °C for 20 min. DNase I-treated total RNA (1 µg) was incubated with avian myeloblastoma virus reverse transcriptase with random primers in 20 µl of a reaction mixture (AMV reverse transcription system; Promega) and then first-strand cDNA was synthesized. The cDNA was amplified using PCR primers for *H. pylori* 16S rRNA, 16SB-F (5′-GCTAAGAGATCAGCCTATGTCC-3′) and 16SB-R (5′-TGGCAATCAGCGTCAGGTAATG-3′) (Engstrand *et al.*, 1992). The *G3PDH* gene on cDNA derived from Mongolian gerbils was amplified using the primers G3PDH-F (5′-ACCACAGTCCATGCCATCAC-3′) and G3PDH-R (5′-TCCACCACCCTGTTGCTGTA-3′) as a non-infected control for total RNA extraction and for standardization of target gene transcriptional activity. Quantification using SYBR Green staining was based on the PCR kinetics of samples expressing various levels of target genes and from comparisons with standard curves that were generated by amplifying serial dilutions of a known quantity of amplicons. With each primer set, PCR was performed in parallel reactions using different amounts of *H. pylori* strain TK1402 chromosomal DNA. Data were analysed using 7500 quantification software (Applied Biosystems) in which background fluorescence was removed by manually setting a noise band.

ELISA. Whole antigen of *H. pylori* for ELISA was prepared based on our previous report (Nakagawa *et al.*, 2005). We cultured *H. pylori* TK1402 on BHS-agar at 37 °C for 3 days and then whole organisms were suspended in 0.01 M PBS (pH 7.4) and disrupted using an ultrasonic Sonifier 250 (Branson Ultrasonics) for 5 min at 20 kHz. The supernatant was separated from the insoluble cells by centrifugation. Microtitre plates (Greiner Labortechnik Japan) were coated at 4 °C for 18 h with whole sonicate *H. pylori* antigens (3 µg per well) and then washed three times with PBS. Antigens were blocked with PBS containing 1 % skim milk (PBS-S; Yukijirushi Nyugyo) at 37 °C for 1 h. Serum samples from infected or uninfected gerbils were added to each well, washed with PBS and then diluted 300-fold with PBS-S. Milk samples were diluted fivefold in PBS. Diluted serum and milk samples (100 µl) were added to the plates and incubated at 37 °C for 2 h, washed three times with PBS, and then 100 µl horseradish peroxidase–protein G (Sigma) at 25 µg ml^{-1} in PBS-S was added to the plates and reacted with antigen–antibody (not specific subtype of immunogloblin) complexes at 37 °C for 1 h. The plates were incubated with 0.1 % *o*-phenylenediamine in developing buffer (0.1 M citric acid, 0.07 M sodium phosphate dibasic, 0.035 % H_2O_2) at room temperature for 5 min and then the reaction was stopped by adding 50 µl 1 M H_2SO_4. The A_{490} was measured using a model 550 microplate reader (Bio-Rad).

Immunohistochemical analysis. The stomach and gastric gland including the gastric mucosa obtained from pups delivered by infected female Mongolian gerbils were fixed in Carnoy's solution (ethanol/chloroform/acetic acid, 6:3:1) at 4 °C for 48 h, cleared in xylene and embedded in paraffin. Mouse mAb HIK1083 specific for *N*-acetylglucosamine-α (GlcNAcα) was purchased from Serotec. Paraffin sections (5 µm thick) were immunostained using mAb

HIK1083 and the EnVision System (Dako). Briefly, endogenous peroxidase activity was blocked with 0.3 % H_2O_2 and then the tissue was sequentially incubated with the mAb HIK1083 and horseradish peroxidase-labelled polymer bound goat anti-mouse antibody. Positive staining was visualized using 3,3′-diaminobenzidine tetrahydrochloride in imidazole buffer containing H_2O_2 and all sections were counterstained with haematoxylin and eosin.

RESULTS AND DISCUSSION

Detection of *H. pylori* in gastric mucosa by culture method and real-time RT-PCR

We cultured the gastric mucosa of gerbil pups aged from 0 to 22 weeks to determine the timing of infection from *H. pylori*-infected mothers. No *H. pylori* colonies were isolated from the gastric mucosa of these animals.

To investigate the timing of vertical *H. pylori* infection, we collected total RNA from the gastric mucosa of pups from infected and uninfected mothers and assembled a complementary DNA library using the reverse transcriptase. Mucosal expression of *H. pylori* 16S rRNA was quantified using real-time RT-PCR with *H. pylori*-specific sequences (Table 1). A product of the 16S rRNA was amplified in mucosa samples from 4–22-week-old pups delivered by infected mothers, but not from uninfected pups. The numbers of *H. pylori* in the gastric mucosa estimated by real-time PCR were 1.3×10^2, 3.4×10^4, 3.1×10^4 and 8.5×10^5 per stomach at 4, 5, 6 and 22 weeks of age, respectively. Direct sequencing of the 16S rRNA gene showed that the products of real-time PCR were *Helicobacter*-specific sequence (data not shown).

We tried to quantify the number of colonized organisms in the stomach by the routine culture method in addition to the real-time RT-PCR method. With the culture method, many investigators have reported that they could detect 10^4–10^5 c.f.u. of *H. pylori* in the gastric mucosa when 10^7–10^9 c.f.u. of *H. pylori* were inoculated (Matsumoto *et al.*, 1997; Sawada *et al.*, 1999; Watanabe *et al.*, 1998). In contrast, the real-time RT-PCR method detected only low numbers of *H. pylori*. In our case, we could not detect *H. pylori* in the stomach by the routine culture method. However, we could detect *H. pylori*, even 4 weeks after birth, and obtained better sensitivity with the real-time RT-PCR method. It was suggested that the number of *H. pylori* organisms infecting from mother to pups was not enough for detection by the culture method, but was detectable by the real-time RT-PCR method.

Antibody titres in maternal gerbil milk and in sera from pups

Mother's milk is considered to suppress mother-to-child transmission. We therefore used an ELISA to measure titres of antibodies against *H. pylori* in milk collected from the stomachs of 0–3-week-old pups immediately after suckling. Fig. 1(a) shows that the A_{490} of anti-*H. pylori* antibodies ranged from 2.5 to 3.5 in pups from infected mothers within 3 weeks post-partum, whereas these antibodies were undetectable in milk from uninfected mothers at any time.

To determine whether anti-*H. pylori* antibody from maternal gerbils protects pups from *H. pylori* infection, we measured titres of serum antibody against *H. pylori* in pups at various times until 22 weeks post-partum (Fig. 1b). Serum antibody against *H. pylori* was detected in 0–5-week-old pups from infected mothers, but not at any time in pups from uninfected mothers. The maximum antibody titre ($A_{490}=2.867 \pm 0.424$) detected at 3 weeks of age rapidly decreased from 4 weeks of age. These results indicated that anti-*H. pylori* antibodies that developed in offspring were maternally derived.

Although the Ig subclass was not determined for the anti-*H. pylori* antibody in the present study, the results obtained suggest that antibodies against *H. pylori* in gerbil pup serum and mother's milk prevent colonization of *H. pylori* in gastric mucosa during the suckling period.

Determination of *H. pylori* 16S rRNA in the oral scrub and fresh faeces of infected mothers

To understand the route of maternal transmission, we tested oral swabs and fresh faeces from infected mothers for *H. pylori* 16S rRNA at 2 and 4 months after infection using real-time RT-PCR. *H. pylori* 16S rRNA was undetectable at 2 months and detectable in a very low number at 4 months

Table 1. Detection of *H. pylori* in the gastric mucosa of pups of infected mothers estimated by real-time RT-PCR

	Age (weeks)							
	0	1	2	3	4	5	6	22
Mean no. of *H. pylori* (relative no. per stomach)	0	0	0	0	1.3×10^2	3.4×10^4	3.1×10^4	8.5×10^5
No. of pups tested	3	3	3	2	6	9	2	2
No. of *H. pylori* 16S rRNA-positive gerbils	0	0	0	0	2	5	2	2
Positive ratio for *H. pylori* infection (%)	0	0	0	0	33.3	55.6	100.0	100.0
Accumulated positive ratio for *H. pylori* (%)	0	0	0	0	11.8	26.9	32.1	36.7

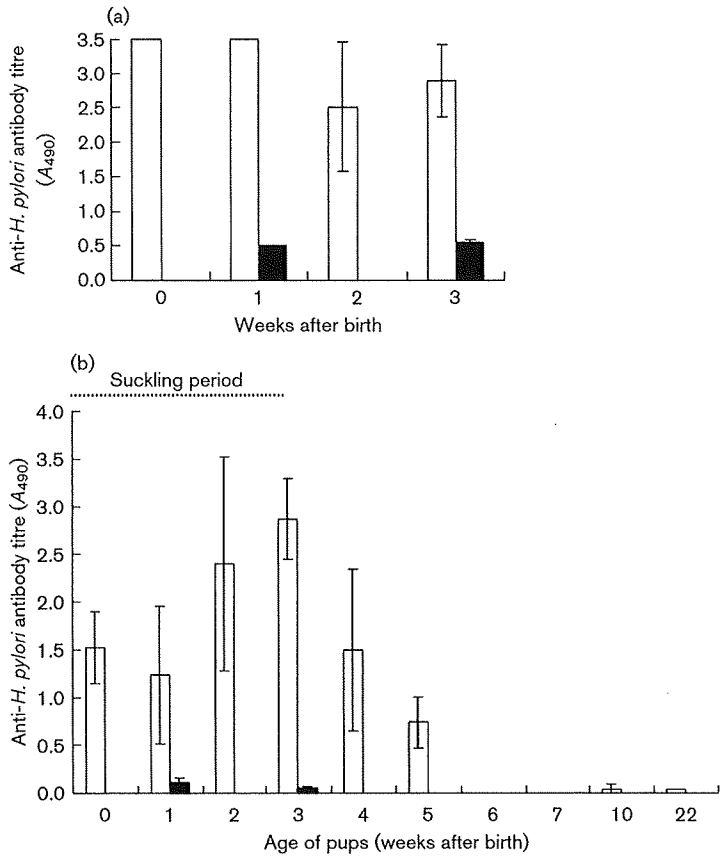

Fig. 1. Antibody titres measured using ELISA in milk from maternal Mongolian gerbils and in sera from pups. (a) Anti-*H. pylori* antibody titres in ingested milk from the stomachs of pups after suckling from infected (□) and uninfected (■) mothers. (b) Anti-*H. pylori* antibody titres in sera from 0-, 1-, 2-, 3-, 4-, 5-, 10- and 22-week-old Mongolian gerbil pups delivered by infected (□) or uninfected (■) mothers. Blood was removed from the hearts of 1–10 sacrificed pups per time point and from the hearts of pups from uninfected mothers at 1 and 3 weeks post-partum.

in oral specimens (Table 2), whereas relatively high numbers of *H. pylori* (10^2–10^3) were detected in the faeces at both time points after infection.

The presence of *H. pylori* 16S rRNA in fresh faeces from infected mothers indicated that live *H. pylori* migrates from the stomach to the intestine. Mongolian gerbil pups have a habit of eating faeces, indicating that faeces-to-mouth transmission was the infection route. If *H. pylori* is transferred through the faeces of an infected person to water for public use and consumption, then the spread of infection could be a concern.

The present results showed that *H. pylori* survives in the faeces of infected mothers, indicating transmission via nappy changes, dish-washing, careless personal hygiene or from public swimming pools. However, detection of the presence of *H. pylori* by real-time RT-PCR in the faeces does not necessarily mean proliferation of *H. pylori*.

Several epidemiological reports have detected *H. pylori* in the oral cavity of infected persons. However, we did not detect *H. pylori* 16S rRNA in the oral cavity of infected Mongolian gerbils. Rodents lack a vomiting reflex (Kuss *et al.*, 2003; Horn *et al.*, 2007), indicating that gastric juice

Table 2. Detection of *H. pylori* by real-time RT-PCR in the oral scrub and fresh faeces from infected mothers

Sample	Months after infection	*H. pylori* relative bacterial no. (g specimen)$^{-1}$ (mean ± SD)
Oral scrub	2	0 ± 0
	4	$1.4 \times 10^0 \pm 2.8 \times 10^0$
Faeces	2	$1.5 \times 10^3 \pm 8.6 \times 10^2$
	4	$7.6 \times 10^2 \pm 6.8 \times 10^2$

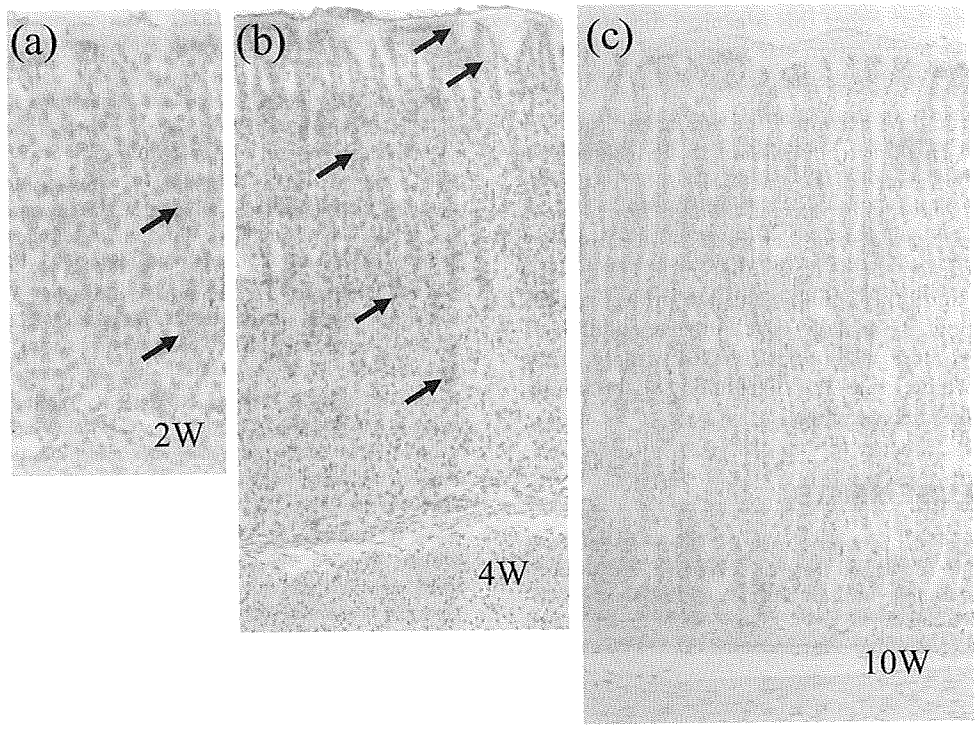

Fig. 2. Expression of GlcNAcα in gastric mucosa of Mongolian gerbil pups delivered by *H. pylori*-infected mothers. GlcNAcα (arrows) is expressed in gastric mucosa of gerbil pups at 2 (a) and 4 (b) weeks, but not at 10 weeks (c) post-partum.

in Mongolian gerbils cannot flow back from the stomach, and thus *H. pylori* probably does not migrate from the stomach to the oral cavity of these animals.

Immunohistochemical analysis of gastric mucosa of pups from infected mothers

GlcNAcα in gastric mucus constitutes an important defence against *H. pylori* infection. We thus immunohistochemically analysed the gastric mucosa of 2–10-week-old gerbil pups using HIK1083, an anti-GlcNAcα mAb. HIK1083 reacted weakly with the immature gastric mucosa of 2-week-old suckling pups (Fig. 2). In contrast, the gastric mucosa of 4-week-old weaned pups was intensively stained with HIK1083. However, the gastric mucosa of 10-week-old pups did not react with HIK1083.

The sugar chain of the gastric mucin GlcNAcα has an inhibitory effect on growth of *H. pylori* (Kawakubo *et al.*, 2004). We found that the anti-*H. pylori* titre in sera of pups from infected mothers increased at 2–4 weeks of age, and then decreased after 4 weeks of age. These results suggest that the development of GlcNAcα from 2 to 4 weeks post-partum suppresses the growth of *H. pylori*. Real-time RT-PCR showed that the estimated numbers of *H. pylori* increased from 4 to 22 weeks of age. This is considered to be partly due to decreased GlcNAcα expression, which confers an environment conducive to *H. pylori* proliferation. Levels of GlcNAcα increased at 4 weeks of age, thus providing defence against *H. pylori* infection, but after GlcNAcα expression decreased, the bacterial cells started to proliferate. Therefore, whether or not *H. pylori* can persist in the gastric mucosa for 4 weeks after birth is critical to its future colonization.

Minoura *et al.* (2005) have speculated that the components of maternal milk such as lactoferrin, glycoconjugates and secretory IgA inhibit bacterial growth, and that the numbers of colonizing bacteria in the stomach are reduced while breast-feeding. We found in this study that suckling pups were not infected, due to a high anti-*H. pylori* antibody titre in the mother's milk during this period. Therefore, anti-*H. pylori* antibody in mother's milk might also contribute to defending gerbil pups against *H. pylori* infection.

Nude mice become infected with *H. pylori* when housed in cages that allow faeces to be consumed, but not when kept in cages that prevent this behaviour (Yoshimatsu *et al.*, 2000; Karita *et al.*, 2005). Transmission of *H. pylori* from faeces to mouth was suggested as the infection route, as *H. pylori* has been cultured from saliva. We detected *H. pylori* rRNA in faeces from infected mothers, supporting the notion of a faecal–oral route of transmission. We did not detect *H. pylori* in the oral cavity of infected maternal

gerbils, indicating that either this organism did not reach the oral cavity, or proliferation was suppressed by salivary antibodies. Further studies are required to clarify this issue.

In the present study, we examined the timing and route of mother-to-child *H. pylori* transmission in the Mongolian gerbil model. We detected *H. pylori* 16S rRNA in the gastric mucosa of pups delivered by infected mothers from 4 to 22 weeks post-partum, suggesting that mother-to-child transmission occurred later than 4 weeks after birth. The anti-*H. pylori* antibody in mother's milk seemed to protect against *H. pylori* infection. By analogy with humans, children of infected mothers are probably not infected with *H. pylori* while breast-feeding, but the likelihood of becoming infected increases after weaning.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Japanese Ministry of Culture, Science and Sports, and Odawara Research Center, Nippon Soda Co. Ltd. The authors thank Dr Shigehito Nakagawa of Sanofi-Aventis K.K. for valuable advice and discussion about the study, and Mr Taki Manzoku of Miyarisan Pharmaceutical Co. Ltd for excellent technical support.

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Journal of Medical Microbiology (2009), 58, 697–705 DOI 10.1099/jmm.0.007872-0

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*, 10^4 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.

Received 21 December 2008
Accepted 27 January 2009

INTRODUCTION

Mycoplasmal 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 extraplumonary lesions (Fernald *et al.*, 1975).

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

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

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 microorganisms, 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 % CO_2 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 Expor (Alcide). The mice were fed a ^{60}Co-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 % CO_2, 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 μg *M. pneumoniae* partially purified antigens and 2 μg gentamicin (Schering-Plough) was added to each well. MOLT-4 cells were incubated at 37 °C under an atmosphere of 5 % CO_2 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 μl) of the M129 strain for the first infection was 1.8×10^5 c.f.u. per mouse. The inoculum (30 μl) of *M. pneumoniae* soluble antigen and partially purified antigens for the first inoculation was 5 μg per mouse. The second inoculation was carried out 4 weeks after the primary inoculation by the same method. A total of 30 μl 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 % CO_2. 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% CO_2. 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% CO_2 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

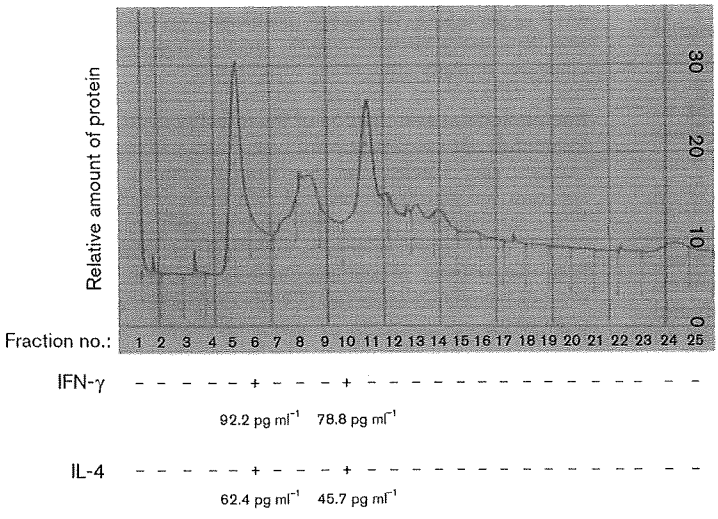

Fig. 1. Purification of antigen from *M. pneumoniae* soluble antigen by FPLC using Superdex 200. Each fraction was used for ELISA to evaluate induction activity of IL-4 and IFN-γ secretion from MOLT-4 cells.

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).

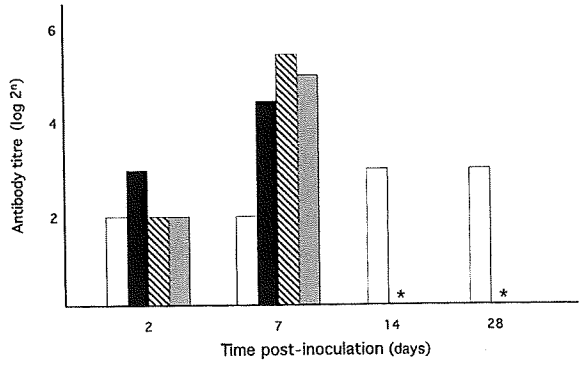

Fig. 2. Anti-*M. pneumoniae* antibody titre measured by particle agglutination. Each bar indicates the mean of the anti-*M. pneumoniae* antibody titre ($n=2$). White bars, *M. pneumoniae*; black bars, sonicate; hatched bars, F6; grey bars, F10. *, Antibody titre was not measured in the germ-free mice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

***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).

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.

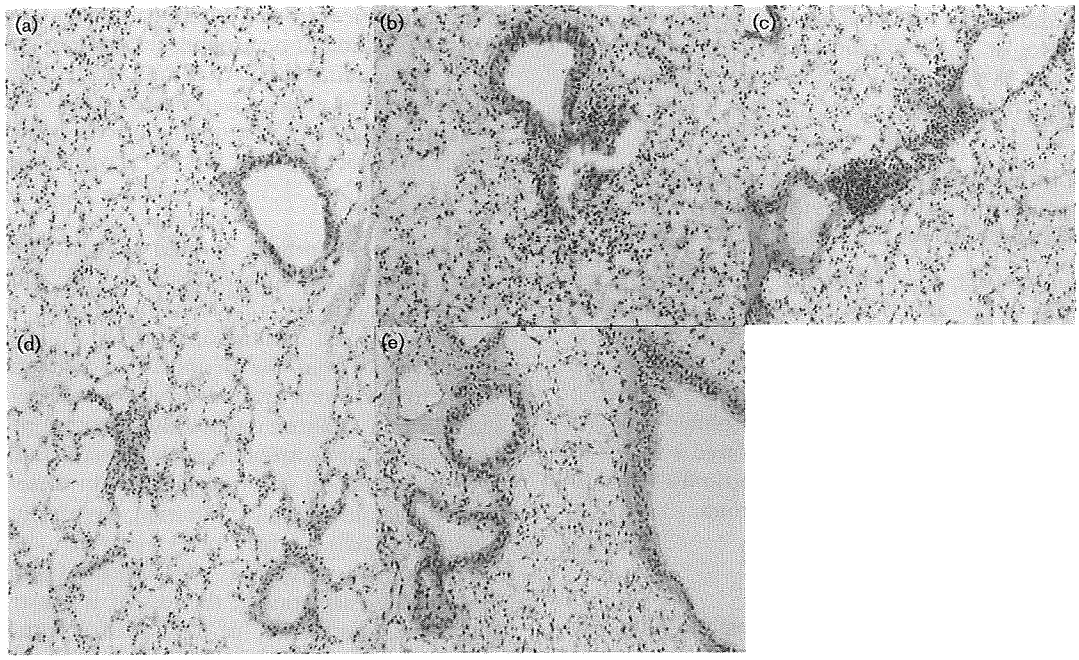

Fig. 3. Histopathological findings for the lung tissue. Lung was fixed and stained with haematoxylin and eosin after intranasal infection of mice with *M. pneumoniae* strain M129 (magnification ×200). (a) Uninfected control, (b) 2 days post-infection, (c) 7 days post-infection, (d) 14 days post-infection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

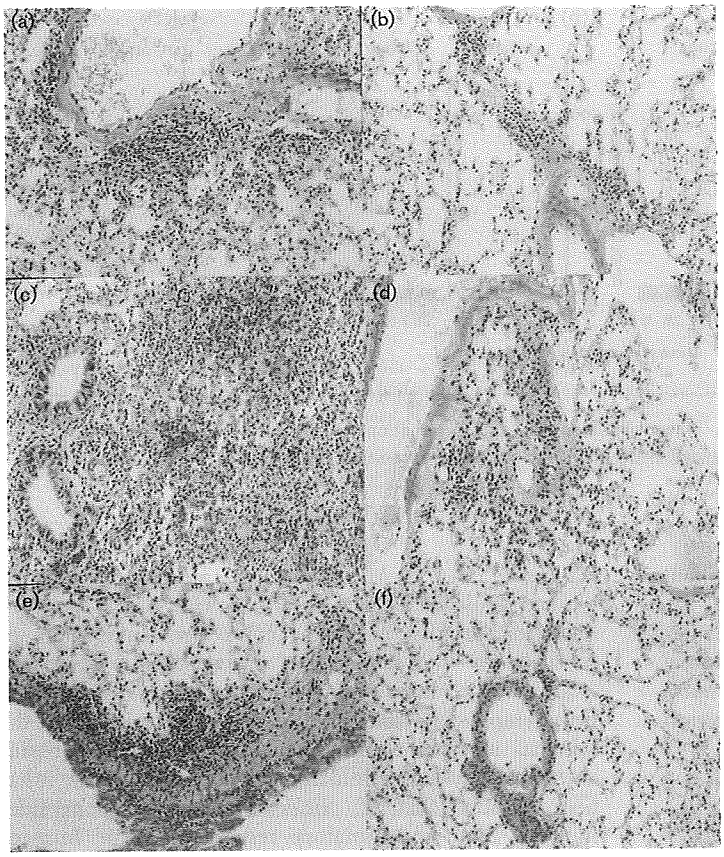

Fig. 4. Histopathological findings for the lung tissue of the germ-free mice inoculated with *M. pneumoniae* soluble antigens (a, b), and the partially purified antigens F6 (c, d) and F10 (e, f). The lung was fixed and stained with haematoxylin and eosin after intranasal inoculation (magnification ×200).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

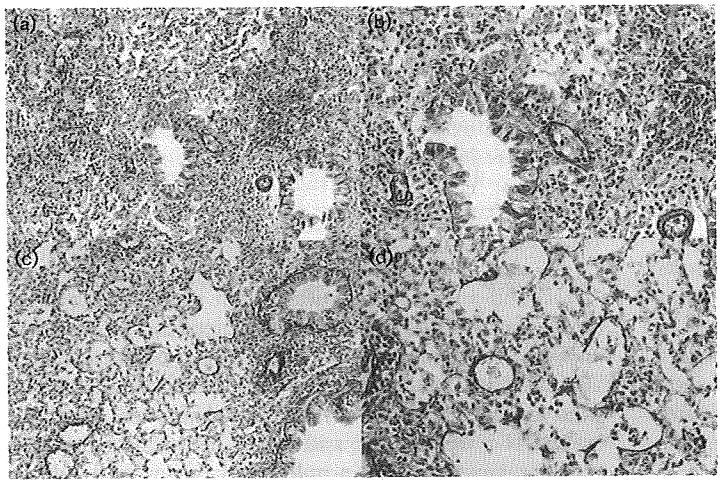

Fig. 5. Histopathological findings for the lung tissue of germ-free mice inoculated with partially purified antigen F6. The lung was fixed and stained with Elastica Masson and Elastica van Gieson stain 2 days after intranasal inoculation with the partially purified antigen F6 of *M. pneumoniae* strain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

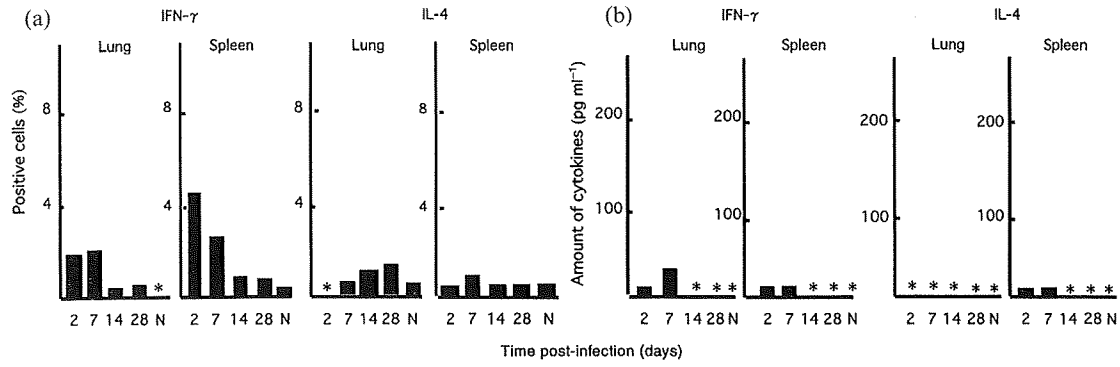

Fig. 6. Positive percentages of intrinsic cytokines in lymphocytes (a) and production of cytokines from lymphocytes (b) recovered from the lung and the spleen of gnotobiotic mice infected with *M. pneumoniae* strain M129. N, Non-infected; *, below the detectable limit.

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

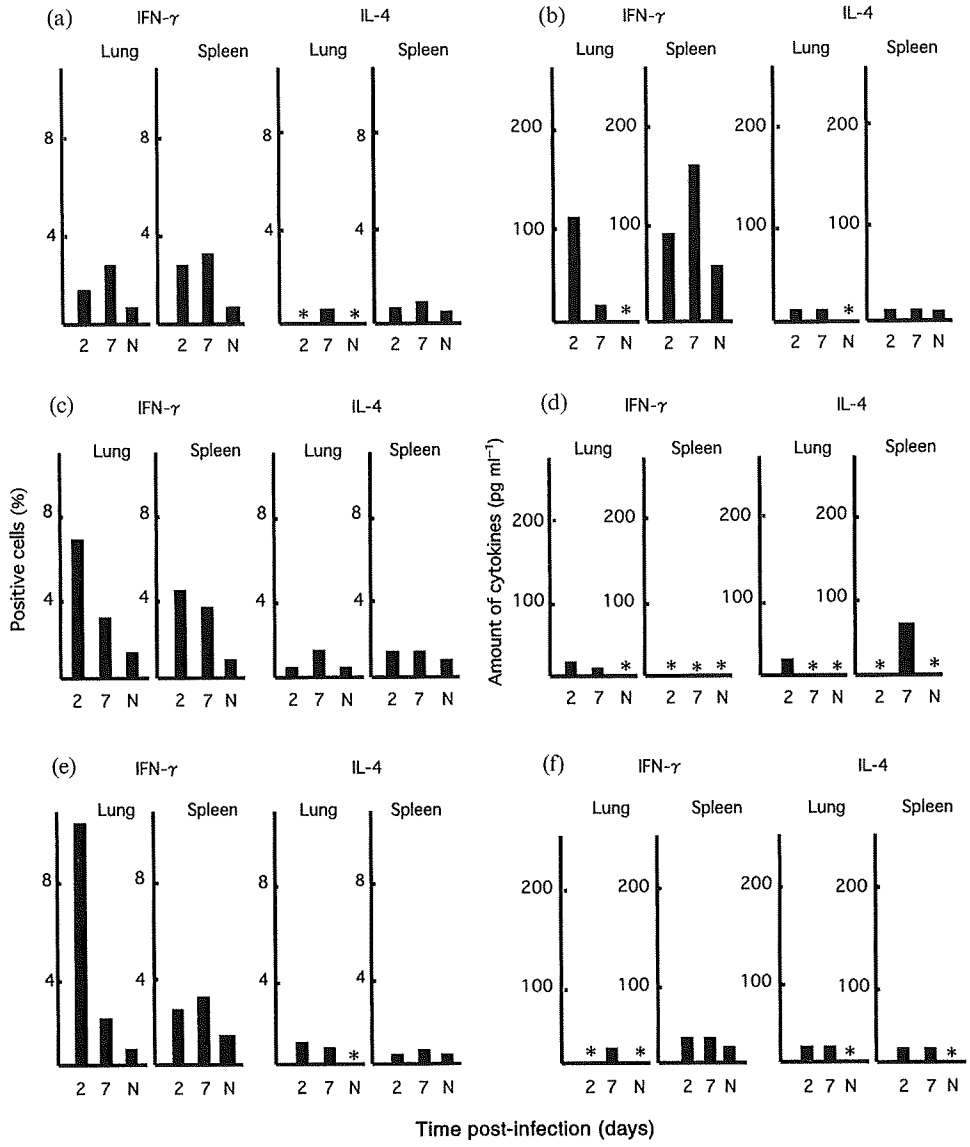

Fig. 7. Positive percentages of intrinsic cytokines in lymphocytes (a, c, e) and production of cytokines from lymphocytes (b, d, f) recovered from the lung and the spleen of germ-free mice. Mice were inoculated with *M. pneumoniae* soluble antigen (a, b), partially purified antigen F6 (c, d) and partially 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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International Journal for Parasitology 40 (2010) 101–108

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

IL-10 plays a crucial role for the protection of experimental cerebral malaria by co-infection with non-lethal malaria parasites

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ARTICLE INFO

Article history:
Received 5 June 2009
Received in revised form 14 August 2009
Accepted 17 August 2009

Keywords:
Experimental cerebral malaria
Co-infection
IL-10

ABSTRACT

Cerebral malaria is an infrequent but serious complication of *Plasmodium falciparum* infection in humans. Co-infection with different *Plasmodium* species is common in endemic areas and the existence of benign malaria parasites, such as *Plasmodium vivax*, during *P. falciparum* infection has been considered to reduce the risk of developing pathogenesis. However, it is still unknown how disease severity is reduced in the host during co-infection. In the present study, we investigated the influence of co-infection with non-lethal malaria parasites, *Plasmodium berghei* (*Pb*) XAT strain, on the outcome of *Pb* ANKA strain infection which causes experimental cerebral malaria (ECM) in mice. The co-infection with non-lethal *Pb* XAT suppressed ECM caused by *Pb* ANKA infection and prolonged survival of mice. The production of TNF-α and IFN-γ, which had been shown to be involved in development of ECM, was suppressed in co-infected mice early in infection. The suppression of ECM by co-infection with *Pb* XAT was abrogated in IL-10-deficient mice. IL-10 plays a crucial role in the suppression of ECM by co-infection with non-lethal malaria parasites, probably due to its suppressive effect on the induction of TNF-α and IFN-γ. Co-infection with *Pb* XAT and *Pb* ANKA is a useful model for understanding how ECM is suppressed.

1. Introduction

Malaria is the infectious disease that causes an estimated 2–3 million deaths and 300–500 million clinical cases globally per annum (Snow et al., 1999). Infection with *Plasmodium falciparum* is associated with developing fever, a high number of parasites in the blood and pathogenesis, including cerebral malaria in humans (Breman, 2001). The development of cerebral malaria is associated with sequestration of parasitized red blood cells (pRBCs) and/or leukocytes within cerebral microvessels (Coltel et al., 2004; Miller et al., 2002; Schofield and Grau, 2005) and high levels of inflammatory cytokines such as TNF-α and IFN-γ during malaria infection (Engwerda et al., 2005; Hunt and Grau, 2003). In endemic areas, co-infection with different *Plasmodium* species is revealed using sensitive PCR-based techniques (Imwong et al., 2005; Siribal et al., 2004). In particular, the simultaneous presence of benign malaria parasites such as *Plasmodium vivax* during *P. falciparum* infection has been shown to reduce the risk of developing a high number of parasites in the blood and pathogenesis (Luxemburger et al., 1997; Mason et al., 1999; Mason and McKenzie, 1999; McQueen and McKenzie, 2006).

Plasmodium berghei (*Pb*) ANKA strain infection in mice is a murine model of cerebral malaria that allows detailed examination of the immune response in vivo (Yañez et al., 1996). Mice infected with *Pb* ANKA demonstrate similar features, neurological symptoms and histopathological results, such as hemorrhages and sequestration within cerebral microvessels, to human cerebral malaria. IFN-γ-deficient (Amani et al., 2000) and TNF-α/β-deficient mice (Rudin et al., 1997) are protected from the development of experimental cerebral malaria (ECM), although the role of TNF-α in ECM is controversial (Engwerda et al., 2002). Co-infection with two different species and/or strains of murine malaria parasites has been shown to influence the parasitemia or mortality of each species and/or strain (Snounou et al., 1992). The development of ECM caused by *Pb* ANKA was inhibited by the simultaneous presence of *Plasmodium yoelii yoelii* or *Pb* K173 strain (Mitchell et al., 2005; Voza et al., 2005). However, it is still unknown how ECM is suppressed during co-infection.

In a previous study, we showed that simultaneous infection with non-lethal *Pb* XAT strain suppressed liver injury caused by *Pb* NK65 strain infection, and that the suppression of liver injury by co-infection was reduced in IL-10-deficient mice (Niikura

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doi:10.1016/j.ijpara.2009.08.009

et al., 2008). To establish the mechanisms by which ECM is exacerbated or suppressed in hosts during co-infection, we have used the non-lethal *Pb* XAT and lethal *Pb* ANKA co-infection system. Firstly, we investigated the influence of co-infection with *Pb* XAT on the outcome of *Pb* ANKA lethal infection, and then examined how *Pb* XAT non-lethal infection modulated the immune responses such as cytokine production during *Pb* ANKA lethal infection. Because inflammatory cytokines such as IFN-γ are shown to be involved in the development of ECM during *Pb* ANKA infection, we also examined whether IL-10 is associated with the reduction of severity by co-infection with *Pb* ANKA and *Pb* XAT.

2. Materials and methods

2.1. Mice

Female C57BL/6 (B6) mice were purchased from CLEA Japan INC (Tokyo, Japan) and used at 5–6 weeks of age. IL-10-deficient mice on a B6 background were purchased from Jackson's Laboratory (Bar Harbor, USA). We used 5- to 6-week-old male or female IL-10-deficient mice. The genotype of IL-10-deficient mice used in experiments was verified by PCR. The experiments were approved by the Experimental Animal Ethics Committee in the Faculty of Medicine, Kyorin University and all experimental animals were kept in the specific-pathogen-free unit with sterile bedding, food and water at the animal facility.

2.2. Parasites and infections

Malaria parasites, *Pb* ANKA or *Pb* XAT, were stored as frozen stocks in liquid nitrogen. *Pb* ANKA is a high-virulence strain and the parasites, which had been cloned by limiting dilution, were obtained from Dr. W.P. Weidanz (University of Wisconsin-Madison, Madison, WI, USA). *Pb* XAT is a low-virulence derivative from *Pb* NK65 (Waki et al., 1982). pRBCs of *Pb* ANKA or *Pb* XAT were generated in donor mice inoculated i.p. with each frozen stock of parasites. The donor mice were monitored for parasitemia daily and bled for experimental infection in ascending periods of parasitemia. Experimental mice were infected i.v. with 1×10^4 pRBCs of a given parasite species or strain. Co-infected mice were infected with *Pb* XAT 1 day before infection with *Pb* ANKA. Therefore, when mice were co-infected with two strains of parasites, a total of 2×10^4 pRBCs (1×10^4 of each parasite strain) were inoculated.

2.3. Parasitemia

pRBCs were observed by microscopic examination of methanol-fixed tail blood smears stained for 45 min with 1% Giemsa diluted in phosphate buffer, pH 7.2. The number of pRBCs in 250 RBCs was enumerated when parasitemia exceeded 10%, whereas 1×10^4 RBCs were examined when mice showed lower parasitemia. The percentage of parasitemia was calculated as follows: [(number of pRBCs)/(total number of RBCs counted)] × 100.

2.4. Measurement of body weights, hematocrits and crisis-form parasites

Body weights were measured using a balance for animals (KN-661; Natume, Tokyo, Japan) and body weight loss was expressed as a percentage of the day 0 value. For hematocrit measurement, tail blood (50 μl) was collected into a heparinized capillary tube and centrifuged at 12,000g for 5 min with a micro-hematocrit centrifuge (HC-12A; Tomy, Tokyo, Japan). The hematocrit value was expressed as a percentage of the total blood volume. The number of pRBCs including crisis-form parasites, which had been defined as morphologically moribund parasites (Jensen et al., 1987), in 1×10^4 RBCs was counted and the percentage of crisis-form parasites was calculated as follows: [(number of pRBC including crisis-form parasites)/(total number of RBCs counted)] × 100.

2.5. Histological examination and cytokine assay

When infected mice showed neurological signs such as a cerebral paralysis, we considered that the mice suffered from ECM and they were euthanized before brains were removed. Their brains were fixed in 10% buffered formalin and embedded in paraffin. Six-μm-thick sections were stained with H & E. Blood was obtained from infected mice and co-infected mice on day 6 p.i. with *Pb* ANKA, and centrifuged at 500 *g* for 10 min. ELISA for the detection of TNF-α or IFN-γ in plasma was carried out as described previously (Kobayashi et al., 2007). A hamster anti-mouse TNF-α monoclonal antibody (mAb) (clone TN3–19.12; eBioscience, San Diego, CA, USA) and a rat anti-mouse IFN-γ mAb (clone R4–6A2; eBioscience) were used as the capture antibodies, and a biotinylated rat anti-mouse TNF-α Ab (eBioscience) and rat anti-mouse IFN-γ mAb (clone XMG1.2; eBioscience) were used as the detecting antibodies. The concentration of cytokines in plasma was calculated from standard curves prepared with known quantities of murine recombinant TNF-α(eBioscience) and murine recombinant IFN-γ (Genzyme, Boston, MA, USA). The limits of detection of these ELISAs were 8 μg/ml for TNF-α and 2.25 U/ml for IFN-γ.

2.6. Detection of cytokine mRNA in spleens

Spleens were removed from infected and co-infected mice on days 3 and 6 p.i. with *Pb* ANKA and total RNA was isolated by Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. The splenic RNA was reverse-transcribed by murine leukemia virus reverse transcriptase (Applied Biosystems, Carlsbad, CA, USA) using random hexamer primers, and the reverse transcriptase reaction was performed at 70 °C for 10 min, at 25 °C for 10 min, and at 42 °C for 30 min. The reaction was terminated by heating at 99 °C for 5 min, and the cDNA products were stored at −20 °C until required. The 50 μl PCR mixture contained 1× TaKaRa Ex Taq buffer, 2.5 mM dNTP, 1 μl cDNA products, 5 U/μl TaKaRa Ex Taq DNA polymerase and 0.25 μM of PCR primers. The primers used for PCR amplification were as follows: IL-10, 5′-GTG AAG ACT TTC TTT CAA ACA AAG, 3′-CTG CTC CAC TGC CTT GCT CTT ATT; β-actin, 5′-CCA GCC TTC CTT CCT GGG TA, 3′-CTA GAA GCA TTT GCG GTG CA. Thirty cycles of PCR were performed on a thermal cycler (iCycler; Bio-Rad, Hercules, CA). Each cycle consisted of 30 s of denaturation at 94 °C, 30 s of annealing at 60 °C and 1 min of extension at 72 °C. The PCR products were analyzed on a 2% agarose gel stained with ethidium bromide.

2.7. Flow cytometry

Flow cytometric analysis was performed on single-cell suspensions of spleen and peripheral blood cells as described previously (Kobayashi et al., 2007). The following mAbs were used for analysis: FITC-conjugated anti-CD3ε mAb (clone 145–2C11; eBioscience); phycoerythrin (PE)-conjugated anti-CD210 (IL-10 receptor; IL-10R) mAb (clone 1B1.3a; BD Pharmingen, San Diego, CA, USA) were added to cells in FACS buffer (1% BSA, 0.1% sodium azide in PBS), incubated at 4 °C for 30 min and the cells were washed with cold FACS buffer by centrifugation at 250 *g* for 2 min. After washing with FACS buffer, cells were fixed with 1% paraformaldehyde. Two-color flow cytometry was performed and analyzed with a FACSCalibur (BD Biosciences, San Jose, CA, USA) using FlowJo software (version 7.1.3, for Windows).