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NOTE

Chemokine Gene Expression in Mice during *Orientia tsutsugamushi* Infection

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***Orientia tsutsugamushi*, an obligate intracellular bacterium, is the causative agent of scrub typhus which is histopathologically characterized by inflammatory manifestations. To understand the pathogenesis of scrub typhus, chemokine gene expression in mice after infection with *O. tsutsugamushi* was investigated. The mRNAs that were upregulated included macrophage inflammatory proteins 1α/β (MIP-1α/β), MIP-2, monocyte chemoattractant protein 1, RANTES (regulated upon activation, normal T-cell expressed and secreted), and gamma-interferon-inducible protein 10. Peak expression of these chemokines was observed six days after infection. These responses returned to or approached baseline preinfection levels by eight days after infection. Chemokine profiles in infected mice were well correlated with the kinetics of inflammatory cell infiltration. Thus, *O. tsutsugamushi* appears to be a strong inducer of chemokines which may significantly contribute to the inflammation observed in scrub typhus by attracting and activating phagocytic leukocytes.**

Key words: *Orientia tsutsugamushi*, scrub typhus, chemokine

Scrub typhus (tsutsugamushi disease), caused by an intracellular bacterium *Orientia tsutsugamushi*, is one of the most prevalent febrile illnesses in South Korea (Chang *et al.*, 1990). The disease is characterized by fever, rash, eschar, pneumonitis, meningitis, and disseminated intravascular coagulation, which leads to severe multiorgan failure in untreated cases (Allen and Spitz, 1945; Chi *et al.*, 1997). This bacterium infects a variety of host cells *in vitro* and *in vivo*, including macrophages, polymorphonuclear leukocytes (PMN), lymphocytes, and endothelial cells, where it replicates in the cytoplasm without being surrounded by a phagolysosomal membrane (Rikihisa and Ito, 1979; Ng *et al.*, 1985). *O. tsutsugamushi* causes local inflammations that are accompanied by eschars at the site of infection, which then spread systemically (Burnett, 1980). Inflammation is initiated by *O. tsutsugamushi*-infected macrophages and endothelial cells in the dermis.

Analysis of early immunologic responses to *O. tsutsugamushi* infection in mice showed that macrophage-mediated cellular immunity is essential for resolution of this infection (Nacy and Groves, 1981). Resistance to the lethal effects of acute rickettsial infection is under unigenic dominant control by the *Ric* locus (Groves *et al.*, 1980). Macrophages infiltrate both susceptible (Ric^s) and resistant (Ric^r) mouse strains in response to *O. tsutsugamushi* Gilliam infection (Jerrells and Osterman, 1981; Nacy and Groves, 1981). A resistant mouse strain was reported to have less PMN response to *O. tsutsugamushi* than a susceptible strain. As a result, susceptible mice died within 2 weeks of infection. By contrast, Ric^r strains showed a minimal level of infection over 2 weeks and survived the infection (Jerrells and Osterman, 1981; Nacy and Groves, 1981). Early host inflammatory responses seem to play a key role in determining the fate of hosts infected with *O. tsutsugamushi* (Jerrells and Osterman, 1981; Nacy and Groves, 1981). For these reasons, the regulatory components that determine the quality and magnitude of cellular influx to the site of rickettsial infection need to be analyzed. Proinflammatory cytokines and chemokines (chemotactic cytokines) play an important role in these processes (Baggiolini *et al.*, 1994). However, the *in vivo* expression of chemokines has not been elucidated in the disease caused by *O. tsutsugamushi*.

Proinflammatory cytokines and chemokines are the main factors responsible for the recruitment of distinct leukocytes into inflamed tissues during inflammatory disease (Baggiolini *et al.*, 1994). The interaction of different chemokines with their receptors on leukocytes allows selective activation and chemotaxis of neutrophils, lymphocytes, or monocytes necessary for migration to the

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sites of evolving inflammation. The site-directed immigration of leukocytes into inflamed tissues is provoked by gradients of chemokines that contribute to the adhesion of leukocytes to the vascular endothelium, transendothelial migration, and movement through the extracellular matrix (Imhof and Dunon, 1995). However, the mechanisms by which leukocytes are attracted to lesional sites in *O. tsutsugamushi* infections are still incompletely understood. In particular, little attention has been directed to the chemokine family in the pathogenesis of scrub typhus. In this study, the gene expression of a subset of chemokines in mice during *O. tsutsugamushi* infection was investigated.

Specific pathogen free, female BALB/c mice purchased from SLC Inc. (Japan) were kept in an animal facility located in Cheju National University College of Medicine. All necessary precautions were taken to maintain the mice free of infection from environmental pathogens. All mice used were 6 to 8 weeks old. *O. tsutsugamushi* Karp (American Type Culture Collection) was propagated in monolayers of L-929 cells as described previously (Kim *et al.*, 1993). When more than 90% of the cells were infected, as determined by an indirect immunofluorescent-antibody technique (Chang *et al.*, 1990), the cells were collected, homogenized with a glass Dounce homogenizer (Wheaton Inc., USA), and centrifuged at 520×*g* for 5 min. The supernatant was then recovered and stored in liquid nitrogen until use. The infectivity titer of the inoculum was determined as described previously with modification (Tamura and Urakami, 1981). The ratio of infected cells to the counted number of cells was determined microscopically, and infected-cell counting units (ICU) of the rickettsial sample were calculated as follows: ICU=(total number of cells used in infection)×(percentage of infected cells)×(dilution rate of the rickettsiae suspension)/100. Mice were infected by intraperitoneal injection of 0.1 ml of rickettsial suspension diluted to contain 1,000 ICU of rickettsiae. At various intervals after infection, mice were sacrificed by cervical dislocation, and peritoneal exudate cells (PEC) were harvested by washing the peritoneal cavity twice with 5 ml of cold Hanks balanced salt solution (HBSS, Gibco BRL, USA) containing 10 U/ml heparin. Peritonel lavage (PL) fluid, from the two washings, was pooled, and spun in a centrifuge at 520×*g* for 5 min at 4°C. Cell pellets were washed once with cold HBSS, and resuspended in PBS. Samples were removed for differential cell counts and the remainder of PEC suspension was snap-frozen in liquid nitrogen, and stored at -70°C for RNA analysis. Total cell counts were determined on a hemacytometer. Cytospins were made in a cytocentrifuge (Wescor Inc., USA) and Giemsa-stained for differential cell counting. Routinely, 200 to 300 cells/cytospin were counted differentially in a random fashion. Total RNA was prepared with SV total RNA isolation system (Promega, USA) as specified by the manufacturer and was quantified spectrophotometrically. Total RNA extracted from each sample (1 to 2 μg per sample) was subjected to first-strand cDNA synthesis at 42°C for 15 min in a 20-μl reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 5 mM $MgCl_2$, 0.1% Triton X-100, 1 mM deoxynucleoside triphosphate mixture, 1 U of RNasin per μl, 0.5 μg of oligo$(dT)_{15}$ primer, and 10 U of avian myeloblastosis virus reverse transcriptase (RT) (all from Promega). The cDNA was heated at 99°C for 5 min and diluted with water. The cDNA amounts equivalent to 100 ng of total RNA were then subjected to PCR amplification in a 25-μl reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM deoxynucleoside triphosphate mixture, 1 μM of each primer, and 0.25 U of *Taq* DNA polymerase (all from Promega) in a GeneAmp PCR System 9600 (Per-

Table 1. Primer sequences used in this study

Primer	Sequences[a]	PCR product size (bp)	No. of PCR cycles
MIP-1α	5'-GGT CTC CAC CAC TGC CCT TGC-3' 5'-GGT GGC AGG AAT GTT CGG CTC-3'	357	20
MIP-1β	5'-AAC CCC GAG CAA CAC CAT GAA G-3' 5'-TGA ACG TGA GGA GCA AGG ACG C-3'	390	25
MIP-2	5'-AGT TTG CCT TGA CCC TGA AGC C-3' 5'-CCA TGA AAG CCA TCC GAC TGC A-3'	536	25
MCP-1	5'-TCT CTT CCT CCA CCA CCA TGC AG-3' 5'-GGA AAA ATG GAT CCA CAC CTT GC-3'	582	25
RANTES	5'-CCT CAC CAT CAT CCT CAC TGC A-3' 5'-TCT TCT CTG GGT TGG CAC ACA C-3'	215	30
IP-10	5'-CCT ATC CTG CCC ACG TGT TGA G-3' 5'-GGC GTC GCA CCT CCA CAT AGC T-3'	436	30
IκBα	5'-GGT GAA GGG AGA CCT GGC-3' 5'-GTG GCC ATT GTA GTT GGT-3'	266	30
β-actin	5'-TGG AAT CCT GTG GGA TCC ATG AAA C-3' 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'	349	25

[a]For each primer pair, the sense primer is given above the antisense primer.

kin-Elmer, USA). The reaction mixture was prepared as a master mixture to minimize reaction variation. One PCR cycle consisted of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min. The PCR products (5-μl samples) were electrophoresed in a 2% agarose gel containing 0.5 μg of ethidium bromide per ml. A 100-bp DNA ladder (Promega, USA) was used at 1 μg/lane as molecular size markers to provide bands from 100 to 1,500 bp. Amplified DNA fragments in the gels were identified according to their size predicted by cDNA sequences. The densities of the bands were analyzed using a Gel Doc 2000 Gel Documentation System and Quantity One software (Bio-Rad, USA). The densitometric intensity was normalized by comparing the densities of chemokine bands with that of β-actin. PCR was performed for the number of cycles detailed in Table 1 for each set of primers to ensure that the assay was in the linear range according to the amount of template (data not shown).

Inflammation in response to intraperitoneal infection with Karp strain rickettsiae was evidenced by an influx of cells into the peritoneal cavity beginning two days after infection (Fig. 1). The cellular influx continued to increase through day 10. In all experiments, injection of BALB/c mice with 1,000 ICU of Karp resulted in 100% mortality in 11 to 12 days (data not shown). To characterize the cellular responses to peritoneal rickettsial infection, a quantitative evaluation was carried out by examining the differential cell types in PL fluid recovered from the peritoneal cavities of infected mice. A rapid increase in the degree of PMN infiltration was observed as early as day 2 of infection. In contrast, the influx of macrophages was delayed until day 6. The influx of these

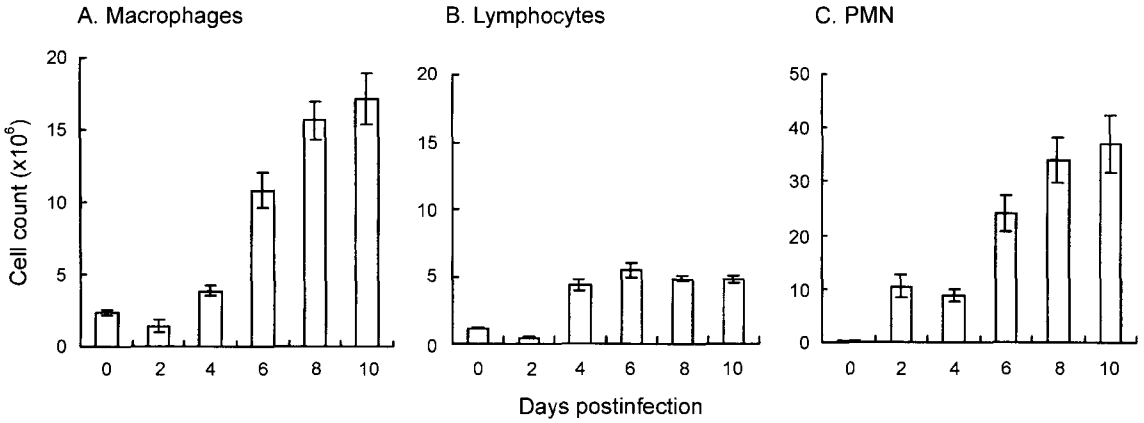

Fig. 1. Peritoneal inflammatory response of BALB/c mice to *O. tsutsugamushi* Karp infection. Total peritoneal cells were recovered from PL fluids collected at various time points postinfection, and differentials were determined on cytospins. Cell counts were calculated from total cell count and differential data. Results are expressed as means±SD of five mice per time point.

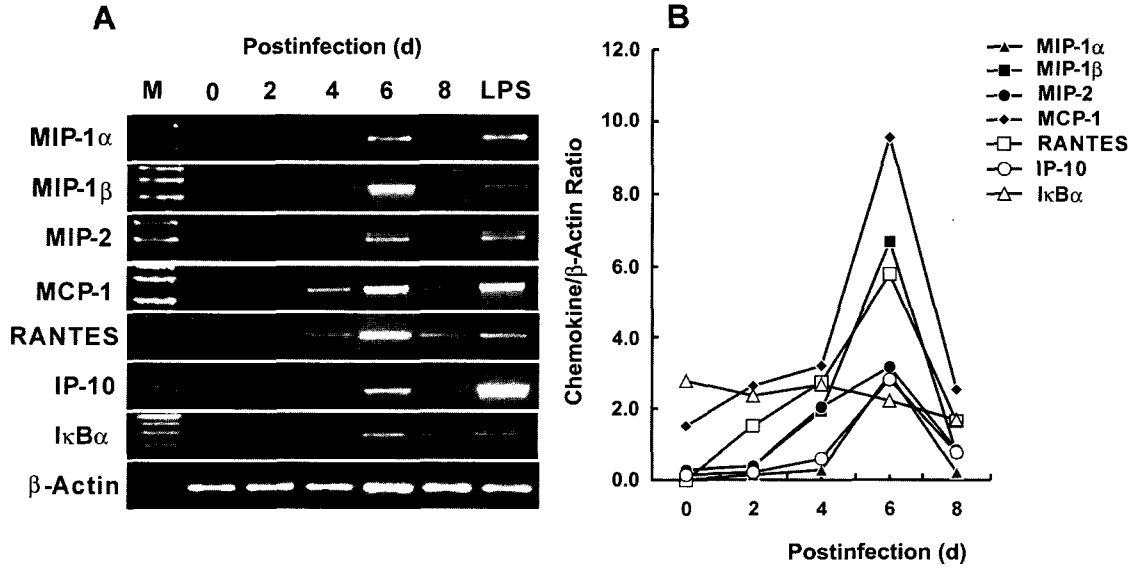

Fig. 2. Chemokine mRNA expression in PEC removed from BALB/c mice after infection with *O. tsutsugamushi* Karp strain. (A) At various times after infection, total RNA was extracted from PEC harvested by peritoneal lavage, and mRNA expression was determined by RT-PCR. M, 100-bp DNA ladder. (B) Densitometric band intensities were determined using Quantity One software, and normalized with mRNA level of β-actin.

cells persisted until death of the mice. The number of infected cells in PL fluid from the infected mice was determined by using an indirect immunofluorescent-antibody technique. It is evident that rickettsiae could be demonstrated within PEC of infected mice by day 4 and that the percentage of infected cells increased until death of the mice (data not shown). Before and after infection of mice with *O. tsutsugamushi*, the levels of chemokine transcripts in PEC were assayed at each time point by semiquantitative RT-PCR (Fig. 2). The mRNAs of the chemokines MIP-1α, MIP-1β, MIP-2, RANTES, and IP-10 were undetectable in uninfected mice, but the MCP-1 transcript was constitutively expressed at low levels in uninfected mice. Transcripts for these chemokines were up-regulated and detected as early as 2 to 4 days after infection, peaked at day 6, and then began to decrease or approach preinfection levels by day 8. IκBα, which is not related to the chemokines, was essentially unchanged over the 8-day period infection.

It has been well documented that macrophages play a pivotal role in early immune responses to *O. tsutsugamushi* infection (Nacy and Meltzer, 1979; Nacy and Grove, 1981; Nacy and Meltzer, 1984). Although inactive tissue macrophages could support the growth of *O. tsutsugamushi* at the site of infection, subsequent cellular influxes, especially of activated macrophages and lymphocytes, have been suggested to be important in protection against *O. tsutsugamushi* infection (Jerrells and Osterman, 1981). Early PMN responses seem to provide a cellular population for rickettsial replication instead of providing antirickettsial activity *in vivo* (Jerrells and Osterman, 1981). Cellular recruitment is controlled largely by chemokines which are secreted by stimulated cells such as macrophages at the site of the primary infection.

This study shows that mice induced the expression of the chemokines MIP-1α, MIP-1β, MIP-2, MCP-1, RANTES, and IP-10 in response to *O. tsutsugamushi* infection. The induction of these chemokine genes peaked at six days after infection. These results correlate well with our previous results, in which the macrophage cell line, J774A.1, showed increased mRNA expression for MIP-1α/β, MIP-2, and MCP-1 in response to *O. tsutsugamushi* infection (Cho *et al.*, 2000). Members of the CC chemokine subfamily, which include RANTES, MIP-1α, MIP-1β, and MCP-1, preferentially attract monocytes and lymphocytes, whereas those of the CXC chemokine subfamily, such as IL-8 and MIP-2, are potent neutrophil attractants (Baggiolini *et al.*, 1994). Thus, the chemokine profiles observed in this study well explain the kinetics of the infiltrations of different kinds of inflammatory cells (Fig. 1).

Protective immunity against *O. tsutsugamushi* is largely due to cell-mediated immune responses, particularly those provided by macrophages and T cells (Nacy and Meltzer, 1979; Jerrells and Osterman, 1982). An explanation for a susceptible/resistant mouse phenotype to *O. tsutsugamushi* infection was provided by an analysis of the early T-lymphocyte activation 1 (*Eta-1*)/osteopontin (*Op*) gene, which maps to the *Ric* locus (Groves *et al.*, 1980; Patarca *et al.*, 1989). The expression of Eta-1 represents an essential early step in the pathway that leads to Th1 immunity by balancing IL-12/IL-10 production (Ashkar *et al.*, 2000). Ric^s mice were found to be defective in the early production of Eta-1 (Patarca *et al.*, 1989). Eta-1/Op is thought to enhance resistance to rickettsial infection by affecting the ability of macrophages to migrate to sites of infection and/or to express bacteriocidal activity (Patarca *et al.*, 1989). In other studies, genetic susceptibility to infectious disease has been shown to be associated with the expression of different cytokine profiles (Heinzel *et al.*, 1995). A correlation between chemokines and a subset of T-cell responses has also been described (Lukacs *et al.*, 1997; Sallusto *et al.*, 1998; Siveke and Hamann, 1998). While the CC chemokines MIP-1α, MIP-1β, and RANTES were found to be efficient chemoattractants for Th1 cells, Th2 cells were not attracted by these chemokines (Siveke and Hamann, 1998). Stimulation of T cells in the presence of MIP-1α was found to enhance IFN-γ production by Th1 cells, while stimulation of T cells in the presence of MCP-1 led to an increased IL-4 production (Lukacs *et al.*, 1997). Thus, the chemokine profiles observed in the present study provoke both Th1 and Th2 cell development and recruitment in the peritoneal cavity of BALB/c mice. This argues against the development of classical polarized Th1- and Th2-type profiles. T-cell cytokine profiles following infection with intracellular bacteria most likely form a continuous spectrum in terms of both levels and combinations, in which polarized Th1- and Th2-type profiles merely represent possible extreme poles of the spectrum (Kelso, 1995).

Based on these studies, I hypothesize that a delicate balance of chemokines exists between the induction of a resistant and a susceptible immune response to rickettsial infection. Further study is required to determine whether quantitative and kinetic differences in the production of chemokines can be correlated with the resistant or susceptible mouse phenotype.

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