The Journal of Microbiology, August 2007, p. 364-366

Vol. 45, No. 4

NOTE

Immunological Roles of *Pasteurella multocida* Toxin (PMT) Using a PMT Mutant Strain

Tae Jung Kim[1†], Nguyen Tat Toan[2†], Eun Jin Jang[2], Bock Gie Jung[2], Jae Il Lee[2], and Bong Joo Lee[1*]

[1]Biotheraphy Human Resources Center, [2]College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Republic of Korea

(Received June 14, 2007 / Accepted July 6, 2007)

The immunological role of the *Pasteurella multocida* toxin (PMT) in mice was examined using a PMT mutant strain. After a nasal inoculation, the mutant strain failed to induce interstitial pneumonia. Moreover, PMT had no significant effect on the populations of CD4+, CD8+, CD3+, and CD19+ immunocytes in blood or on the populations of CD4+ and CD8+ splenocytes ($P<0.01$). However, there was a significant increase in the total number of cells in the BAL samples obtained from the wild-type *P. multocida*-inoculated mice. On the other hand, the level of IL-1 expression decreased when the macrophages from the bronchio-alveolar lavage were stimulated with PMT. Overall, PMT appears to play some role (stimulating and/or inhibiting) in the immunological responses but further studies will be required to confirm this.

Keywords: knock-out, *Pasteurella multocida* toxin (PMT), sub-population, *toxA*

The *Pasteurella multocida* toxin (PMT), which is encoded by the *toxA* gene, is the major virulence factor associated with porcine atrophic rhinitis (AR) (Hunt *et al.*, 2000). PMT is produced by some *P. multocida* serotype A and D strains (Pullinger *et al.*, 2004), and is a very potent mitogen in various cell types, including fibroblasts, osteoblastic cells, and dendritic cells (Rozengurt *et al.*, 1990; Mullan and Lax, 1996; Lax and Grigoriadis, 2001; Lax *et al.*, 2004; Bagley *et al.*, 2005), by modulating the progression of the host cell-cycle (Oswald *et al.*, 2005). PMT is one of the major factors contributing to the pathogenesis of AR, and inhibits osteoblast differentiation and bone formation (Mullan and Lax, 1996). However, the roles of PMT in the immune processes are not completely understood. A *toxA*-deleted mutant strain was produced in a previous study examining the immunological roles of PMT (Kim *et al.*, 2006). With this mutant strain, a series of immunological studies were performed, which included the immunocyte population, cytokine expression levels, and the induction of pneumonia.

In order to better understand the effects of PMT on the proliferation of *P. multocida*, the wild-type (W/T, National Veterinary Research & Quarantine Service, Korea) and mutant (Kim *et al.*, 2006) strains were inoculated on a brain-heart infusion (BHI, BBL™, Becton, Dickinson and Company, USA) broth. Culture samples were taken at 3, 6, 9, 13, 21, and 27 h after inoculation. The number of cells on chocolate agar was calculated, and a growth curve was generated.

† This authors contributed equally to this work.
* To whom correspondence should be addressed.
(Tel) 82-62-530-2850; (Fax) 82-62-530-2857
(E-mail) bjlee@chonnam.ac.kr

BALB/c mice (Charles River Laboratories, Japan) were pre-treated twice with a 1% acetic acid solution 24 h and 6 h before the nasal inoculation. The mice (4 mice for each group) were inoculated with 100 μl (1×10^6 cells/mouse, which corresponds to LD50) of freshly prepared W/T and mutant bacterial cultures. The mice were sacrificed after 7 days inoculation. The lung tissues were formalin fixed and H&E stained for the histology examination.

Each of 5 mice was pre-treated and inoculated with the bacterial cultures, as described above. After 3 days inoculation, the bronchio-alveolar lavage (BAL) samples were collected by inserting a suitable cannula towards the lungs into the exposed trachea. A 2 ml syringe was attached to the cannula and the lungs were washed with 1 ml of phosphate-buffered saline (PBS) containing 1.0% bovine serum albumin (BSA) by flushing the solution in and out of the lungs several times. The wash solutions were collected into a suitable tube. The BAL samples were then washed twice with PBS. The number of total lymphocytes from each group was counted using a hemocytometer. The lymphocytes in the blood were collected from each of 5 mice. The sub-population of lymphocytes, including B, T, and NK cell using anti-CD3, CD4, CD8, and CD19 cell markers (phycoerythrin and/or FITC-conjugated monoclonal antibodies, BD PharMingen, USA) was determined by flow cytometry (FACSCalibur™, BD, USA). Separately, after 2 and 3 days inoculation, the splenocytes were collected and the sub-population of CD4+ and CD8+ cells was calculated. A Student's *t*-test was used to determine the differences in the sub-population of cells. A P value <0.01 was considered significant.

The alveolar macrophages were separated from the BAL samples and used to prepare the total RNA. Briefly, the

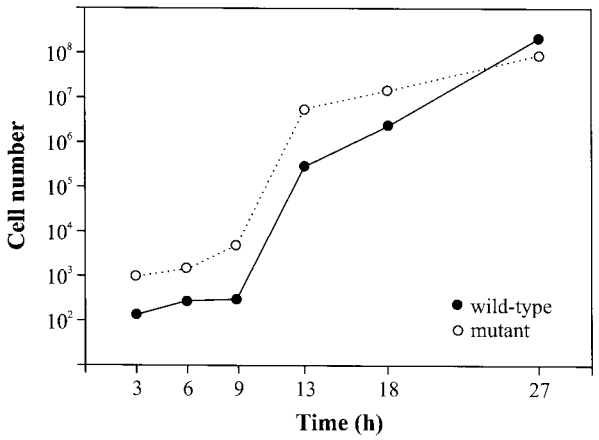

Fig. 1. The effect of PMT on the growth of *P. multocida*. There was no significant difference observed between the wild-type and PMT mutant.

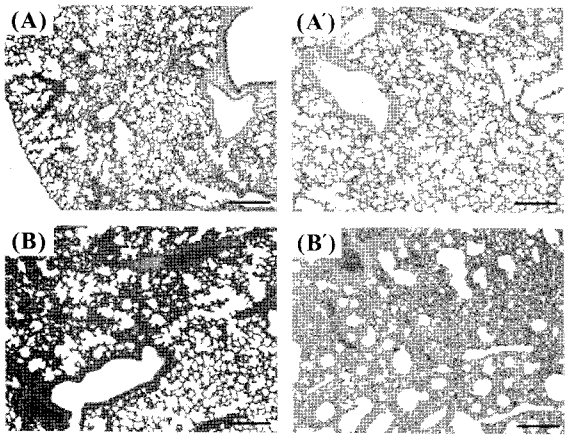

Fig. 2. (A) and (A'): lungs from the mice infected with the PMT mutant strain. No significant regions can be seen. (B) and (B'): lungs from mice infected with the wild type strain. Diffuse moderate to severe interstitial pneumonia findings can be seen (×100). The scale bar represents 200 μm.

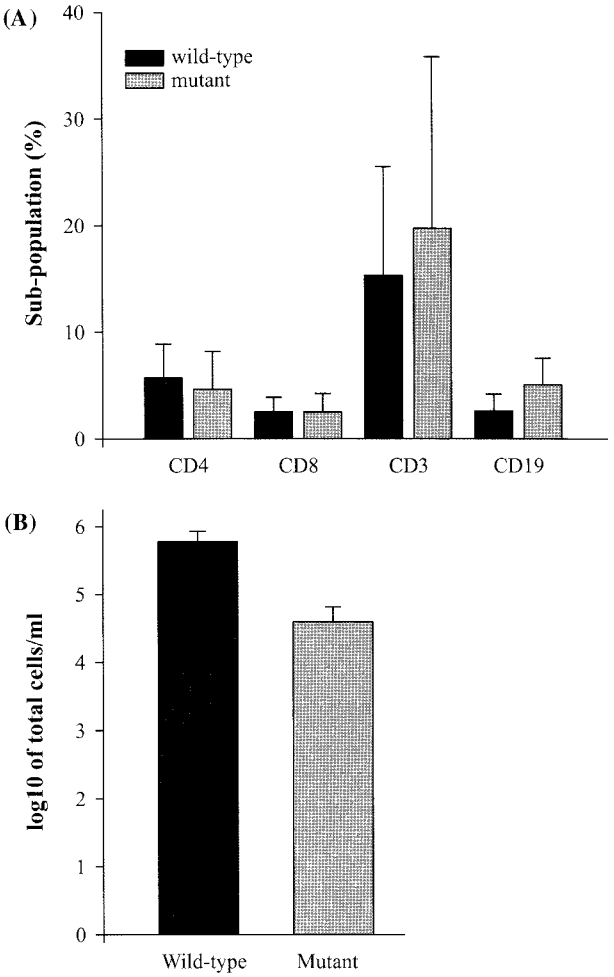

Fig. 3. Effect of PMT on the sub-population of CD4+, CD8+, CD3+, and CD19+ cells in blood (A) and the total number of cells in the BAL (B). (A) Although a slight increase in CD3+ and CD19+ cells was observed in the mutant *P. multocida*-inoculated mice, PMT had no significant effect. (B) A significant increase in the total number of cells in the BAL samples obtained from the wild-type *P. multocida*-inoculated mice.

BAL samples were prepared from 5 healthy, un-treated BALB/c mice, incubated on RPMI1640 media for one night, and washed. The adherent cells (alveolar macrophages) were then harvested. The cells were stimulated using the following procedure. The supernatants of the bacterial culture lysate (Kim *et al.*, 2006) were prepared in a BHI broth. A 50 ml sample of an overnight bacterial culture (W/T and PMT mutant) containing 1×10^8 cells/ml was pelleted, washed twice with 10 ml of PBS, sonicated in 10 ml of PBS. The resulting cultures were pelleted again and the supernatants were filtered through a 0.2 μm membrane filter.

The cells were harvested after 4 h of bacterial lysate stimulation. The total RNA was isolated from the cells using TRIzol® reagent (Invitrogen, USA). The cDNA was synthesized using a SuperScript™ Choice System (Invitrogen), and the levels of IL-1 and IFN-γ mRNA were calculated by RT-PCR. β-Actin was used as the control. The amplified products were visualized on 1.5% agarose gels, and the intensity of the PCR products was compared.

PMT had no influence on bacterial growth because there was no significant difference in the number of cells between the W/T and mutant strains (Fig. 1). The BALB/c mice inoculated with the mutant strain had no signs of pneumonia (Fig. 2A), while the mice inoculated with the W/T exhibited moderate to severe interstitial pneumonia (Fig. 2B). This suggests that PMT is one of the most important pathogenic factors in the induction of pneumonia. Although the T and B cell sub-population was unaffected (i.e. no significant difference, Fig. 3A), the total number of immunocytes was significantly higher ($P<0.01$) in the BAL samples obtained from the W/T inoculated mice (Fig. 3B). This increase is believed to be due to the infiltration of immunocytes in interstitial pneumonia, even though there were no changes in the T and B cell sub-population. After 2 and 3 days of inoculation, the splenocytes were collected from each of 5 mice. There were no differences in the CD4+ and CD8+ cell sub-pop-

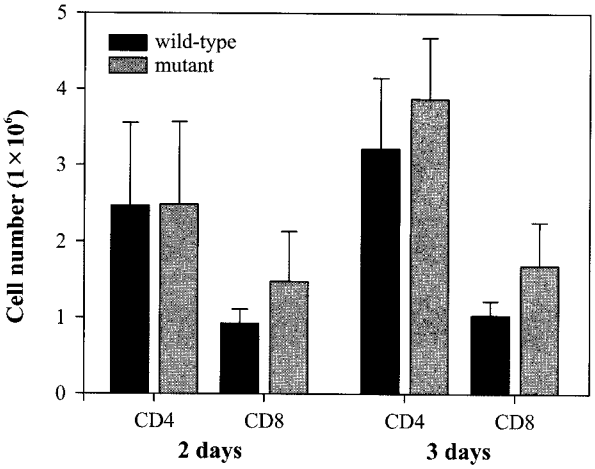

Fig. 4. Effect of PMT on the sub-population of CD4+ and CD8+ cells in the spleen. Although there was a slight increase observed after 3 days inoculation (right), PMT had no significant effect.

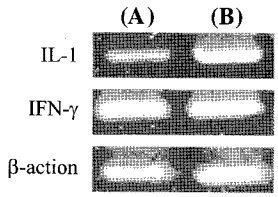

Fig. 5. RT-PCR results of the effects of PMT on the two immune response-related genes, IFN-γ and IL-1. PMT (wild-type *P. multocida*) decreased the level of IL-1 expression (middle B) but had no effect on IFN-γ expression (right).

ulation observed after 2 days of inoculation (Fig. 4, left). Although not significant, there was a slight increase in the samples inoculated with the PMT mutant after 3 days of inoculation (Fig. 4, right). Overall, PMT appears to have no effect on the changes in the sub-population of cells in the blood and spleen. The cDNA level of IL-1 expression decreased when stimulated with W/T (Fig. 5, middle B) but no significant effect on IFN-γ expression was observed (Fig. 5B). The β-actin controls showed no changes (Fig. 5A).

In conclusion, PMT has no effect on the bacterial growth *in vitro*. However, when administered to mice, PMT is believed to play an important role in inducing interstitial pneumonia and has some influence on the immune responses.

The authors acknowledge a graduate fellowship provided by the Korean Ministry of Education and Human Resources Development through the Brain Korea 21 Project.

This work was supported by the "Regional Technology Innovation Program of the Ministry of Commerce, Industry, and Energy (MOCIE), Republic of Korea", the "Korea Research Foundation grant number KRF-2004-041-E00331", and the CNU Specialization Grant funded by Chonnam National University.

References

Bagley, K.C., S.F. Abdelwahab, R.G. Tuskan, and G.K. Lewis. 2005. *Pasteurella multocida* toxin activates human monocyte-derived and murine bone marrow-derived dendritic cells *in vitro* but suppresses antibody production *in vivo*. *Infect. Immun.* 73, 413-421.

Hunt, M.L., B. Adler, and K.M. Townsend. 2000. The molecular biology of *Pasteurella multocida*. *Vet. Microbiol.* 72, 3-25.

Kim, T.J., J.I. Lee, and B.J. Lee. 2006. Development of a *toxA* gene knock-out mutant of *Pasteurella multocid*a and evaluation of its protective effects. *J. Microbiol.* 44, 320-326.

Lax, A.J. and A.E. Grigoriadis. 2001. *Pasteurella multocida* toxin: the mitogenic toxin that stimulates signalling cascades to regulate growth and differentiation. *Int. J. Med. Microbiol.* 291, 261-268.

Lax, A.J., G.D. Pullinger, M.R. Baldwin, D. Harmey, A.E. Grigoriadis, and J.H. Lakey. 2004. The *Pasteurella multocida* toxin interacts with signalling pathways to perturb cell growth and differentiation. *Int. J. Med. Microbiol.* 293, 505-512.

Mullan, P.B. and A.J. Lax. 1996. *Pasteurella multocida*toxin is a mitogen for bone cells in primary culture. *Infect. Immun.* 64, 959-965.

Oswald, E., J.P. Nougayrede, F. Taieb, and M. Sugai. 2005. Bacterial toxins that modulate host cell-cycle progression. *Curr. Opin. Microbiol.* 8, 83-91.

Pullinger, G.D., T. Bevir, and A.J. Lax. 2004. The *Pasteurella multocida* toxin is encoded within a lysogenic bacteriophage. *Mol. Microbiol.* 51, 255-269.

Rozengurt, E., T. Higgins, N. Chanter, A.J. Lax, and J.M. Staddon. 1990. *Pasteurella multocida* toxin: potent mitogen for cultured fibroblasts. *Proc. Natl. Acad. Sci. USA* 87, 123-127.