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Full Length Research Paper

Cytokine gene expression of peripheral blood lymphocytes stimulated by lipopolysaccharide

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Lipopolysaccharide (LPS) is a predominant glycolipid in the outer membranes of gam-negative bacteria that stimulates monocytes, macrophages, and neutrophils to produce cytokines. The aim was to study the expression profile of TLRs and cytokines and determine the role of LPS in the peripheral blood lymphocytes. Lymphocytes were isolated from the peripheral blood of chickens and induced with LPS, total RNA was extracted and reverse transcribed into cDNA. The mRNA expression level of TLRs and inflammatory cytokines were quantitatively determined by semi-quantitative RT-PCR. The result showed that lymphocytes were expressed mRNA of TLRs (TLR1/6/10, TLR3, TLR4, TLR5 and TLR7) and interleukins (IL-1β, IL-8, IL-18 and TGF-β4). LPS will significantly (P<0.05) induce the expression of TLR4, IL-1β, IL-8 and IL-18, and non significantly (P>0.05) induce the mRNA expression of TLR1/6/10, TLR5 and TGF-β4. LPS plays an important role in the innate immune response of lymphocytes to pathogen.

Key words: Lipopolysaccharide, lymphocytes, TLRs, cytokines.

INTRODUCTION

Lipopolysaccharide (LPS), a predominant glycolipid in the outer membranes of Gam-negative bacteria, stimulates monocyte, macrophages, and neutrophils and increase expression of cell adhesion molecules (Trent et al., 2006). LPS is composed of lipid A, core antigen and O antigen; saturated free fatty acids are essential components of lipid A that interact with Toll-like receptor (TLR) and stimulated signaling pathways (Lee et al., 2003; Imler and Zheng, 2004). LPS induces the production of various cytokines and mediators in heterophils such as TNF-α, IL-6, IL-8 and IL-12 (Verhasselt et al., 1997; Kogut et al., 2005). LPS stimulates cells through binding of specific membrane receptors, thereby initiating a cascade of signal transduction events (Diks et al., 2001). The molecules involved in LPS-mediated cell signaling include CD14 and LPS binding protein.

Recognition of potential pathogenic microbes by the innate immune system is the function of pattern recognition receptors (PRRs), which include the TLRs (Kogut et al., 2005; Iqbal et al., 2005; Harris et al., 2006). Toll-like receptors are a family of germline-encoded innate immune receptors that recognize pathogen-associated molecular patterns, for example, bacterial LPS induces signaling through TLR4 (Akira et al., 2006). TLR ligands such as lipopolysaccharide and double-stranded RNA are known to act as adjuvants, enhancing the adaptive immune response (Hoebe et al., 2004). TLR engagement mediates the maturation and migration of dendritic cells to lymph nodes that facilitates interaction with T lymphocytes (Iwasaki and Medzhotow, 2004). TLR expression on B cells can be up regulated when B cells are activated (Bourke et al., 2003). Most studies examining the cellular expression of TLRs on immune cells have focused on neutrophils, monocytes and dendritic cells, but there is little evidence of TLRs being expressed on lymphocytes (Dasari et al., 2005). In the present study the expression profiles of TLR receptors and cytokines in peripheral blood lymphocytes were determined and the role of LPS in the immune response of lymphocytes was quantitatively determined by semi-quantitative RT-PCR.

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Table 1. Sequence of TLR primers used in semi-quantitative RT-PCR.

Primer	Sequence	Accession number
TLR1/6/10 F	5 CGGAAAGCCTATCATTGTCA 3	BQ484541/ BU471924
TLR1/6/10 R	5 TTTGTCTGCGTCCACTGC3	BQ484541/ BU471924
TLR2 Type1F	5 TTAAAAGGGTGTGCCAGGAG3	AB050005
TLR2 Type1R	5 GTCCAAACCCATGAAAGAGC3	AB050005
TLR2 Type2F	5 AGGCACTTGAGATGGAGCAC3	AB046533
TLR2 Type2R	5 CCTGTTATGGGCCAGGTTTA3	AB046533
TLR3 F	5 CCACTCTGGAAGAAAATGAGC3	BI066273
TLR3 R	5 TCATTCTCACCGCTTTTCAG3	BI066273
TLR4 F	5 AGTCTGAAATTGCTGAGCTCAAAT3	AY064697
TLR4 R	5 GCGACGTTAAGCCATGGAAG 3	AY064697
TLR5 F	5 CCACATCTGACTTCTGCCTTT 3	AJ626848
TLR5 R	5 CAGCTAGGGTTACATTGGTTTC 3	AJ626848
TLR7 F	5 GCCTCAAGGAAGTCCCCAGA 3	AJ632302
TLR7 R	5 AAGAAACATTGCATGGATTACGG3	AJ632302
β-actin F	5 TGCTGTGTTCCCATCATCG 3	L08165
β-actin R	5 TTGGTGACAATACCGTGTTCA 3	L08165

F = forward; and R = reverse.

MATERIALS AND METHODS

Experimental animals

Green shell egg chicks were obtained on the day-of-hatch from the hatchery of Poultry Institute, Chinese Academy of Agricultural Science, Yangzhou and placed in floor pens on pine shavings. Birds were provided supplemental heat, water and a balanced, unmedicated chick based ration *ad libitum*.

Lymphocytes isolation

Avian lymphocytes were isolated from the peripheral blood of two-day-old chickens; blood from chickens was collected in tube containing disodium ethylenediaminetetraacetic acid (EDTA) and mixed thoroughly. The blood was diluted 1.5:1 with RPMI-1640 media containing 1% methylcellulose (Sigma-Aldrich, USA) and centrifuged at 1000 rpm for 15 min. The serum and buffy coat layers were retained and suspended in an equal volume of Ca^{2+}, Mg^{2+} free Hanks-balanced salt solution (HBSS). This suspension was layered over a discontinuous Histo-paque 1077 gradient (Sigma-Aldrich, USA). The gradient was then centrifuged at 2000 rpm for 45 min. After centrifugation, the interfaces and band containing the lymphocytes was collected into new tube and washed twice in HBSS and resuspended in fresh RPMI 1640. Cell viability was determined by trypan blue exclusion and the purity of the cell was assessed by microscopic examination of Wright-Giemsa stain smears. The cell concentration was adjusted to $1x10^7$ lymphocytes/ml and stored on ice until used.

Administration of LPS

The lymphocytes (1×10^7 cells/ ml) suspended in RPMI-1640 medium without antibiotics was served as control to establish basal cytokine levels. Ultra-pure lipopolysaccharide (*Escherichia coli*) were purchased from Sigma-Aldrich, USA and prepared in sterile physiological water according to manufacturer instructions. Lymphocytes were treated with (20 µg/ml) lipopolysaccharide in 1.5 ml eppendorf tube for 1 h at 37°C (Kogut et al., 2005).

Isolation RNA and RT-PCR

The total RNA was extracted in the presence of buffer containing β-mercaptoethanol and guanidine using RNAiso plus kit (Takara Biotechnology Dalian, Co. LTD, China) following manufacturer's instructions eluted with 40 µl RNase-free water. The concentration of RNA was measured using a Nano Drop ND-1000 Spectrophotometer (Nano Drop Technologies, Wilmington, USA) and purity (A_{260}/A_{280}) of > 1.8 was used.

250 ng of total RNA from each sample was transcribed into cDNA using the Takara reverse transcription kit (Takara Biotechnology Dalian, Co. LTD, China) according to manufacturer's instructions. Briefly, oligo dT Primer (50 µM) was used to reverse transcribe 250 ng/µg of respective RNA in the presence of dNTPs mixture (10 mM each), 5 X PrimeScript™ buffer, RNase Inhibitor (40 U/µl) and PrimeScript™ RTase (200 U/µl) at 42°C for 60 min following inactivation at 95°C for 5 min.

Polymerase chain reactions (PCR) were performed for Toll like receptors and cytokines according to standard protocols with the primers indicated in Tables 1 and 2. Briefly, cDNA (2 µl) was reacted with 250 mM dNTPs, 1 x reaction buffer (Takara Biotechnology Dalian, Co. LTD, China), forward and reverse primers (5 pM) and 0.4 units Taq polymerase in a 25 µl final reaction volume. PCR conditions were as follows, 1 cycle of 94°C for 4 min followed by 30 cycles of 1 min at 94°C, 55°C for 1 min and 72°C for 1 min followed by 1 cycle at 72°C for 10 min. 7 µl of each PCR product was electrophoresed on a 2% agarose gel (Gene Tech. Shanghai Company limited) in 1xTAE buffer at 60 V for 45 min and visualized with ethidium bromide under UV light (BIORAD).

Statistical analysis

Semi-quantitative analysis was performed by image Quant TL (Amersham Biosciences, USA). Band intensity was expressed as relative absorbance units to β-actin. Significant difference was obtained using a student T-test (Steel and Torrie, 1980), the analysis was performed by SAS software.

Table 2. Sequence of interleukins primers used in semi-quantitative RT-PCR.

Primer	Sequence	Accession number
IL-1 β F	5 GTG GCA CTG GGC ATC AAG GG 3	AJ245728
IL-1 β R	5 CAG GGA GGT GCA GAT GAA C 3	AJ245728
IL-8 F	5 GCC CTC CTC CTG GTT TCA 3	AJ009800
IL-8 R	5 TGC TGG CAT GTA TAA AGA AGA GAG 3	AJ009800
IL-18 F	5 CCA TGC ACA TAA TAC TGA G 3	AJ416937
IL-18 R	5 AGT CGA TTG CTA CAG AAA G 3	AJ416937
TGF-β4 F	5 AAG GAT CTG CAG TGG AAG TGG A 3	M31160
TGF-β4 F	5 CAT TCC GGC CCA CGT AGT AA 3	M31160

F = forward; and R = reverse.

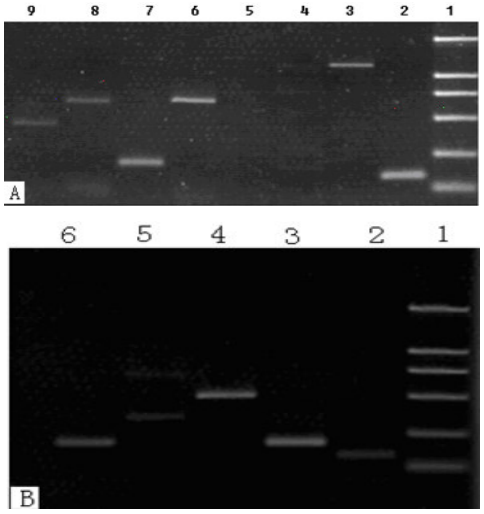

Figure 1. The expression pattern of TLRs and inflammatory cytokines mRNA in avian lymphocytes. A. TLRs mRNA expression in avian lymphocytes. 1, DL2000 Marker; 2, Beta actin; 3, TLR1/6/10; 4, TLR2 type 1; 5, TLR2 type 2; 6, TLR3; 7, TLR4; 8, TLR5; 9, TLR7. B. Cytokines mRNA expression in avian lymphocytes.1, DL2000 Marker; 2, Beta actin; 3, IL-1β; 4, IL-8; 5, IL-18; 6, TGF-β4.

RESULTS AND DISCUSSION

As shown in Figure 1A, peripheral blood lymphocytes expressed TLR1/6/10, TLR3, TLR4, TLR5 and TLR7. Similarly, lymphocytes expressed IL-1β, IL-8, IL-18 and TGF-β4 (Figure 1B). Iqbal et al. (2005) indicated that the expression of chicken TLR2 type 1 and type 2 in tissues was more restricted. The patterns of chicken TLR mRNA expression in immune cell subsets are broadly similar to those detected in mammalian species (Applequist et al., 2002; Zarember and Godowskit, 2002). LPS stimulates cells through binding of specific membrane receptors, thereby initiating a cascade of signal transduction events (Diks et al., 2001). The molecules involved in LPS-mediated cell signaling include CD14, LPS binding protein and TLRs (Iontcheva et al., 2004). Toll receptors were first characterized in *Drosophila melanogaster* and have a role in both embryonic patterning and innate immunity (Belvin and Anderson, 1996). More recently, a related family of TLRs was discovered encoded in vertebrate genomes. Similar to Toll from *Drosophila* species, TLRs have a central role in innate immunity and are also required for the development of an adaptive im-mune response (Akira and Takeda, 2004).

The 20 µg/ml of LPS used in this study was similar to that used by kogut et al. (2005), we induced the cell for 1 h because the mRNA expression of cytokines was increased to 1 h, then the expression was decreased after 1 h of induction time (Swaggerty et al., 2004). The results showed that TLR4 was significantly ($P<0.05$) involved in LPS-mediated cell signaling in avian lym-phocytes (Figure 2A). However, previous studies indicated that TLR1 and/or TLR6 induces signals after exposure to bacterial lipoproteins and peptidoglycans (Takeuchi et al., 2001); LPS from gram-negative bacteria signals through TLR4 (Takeda et al., 2003), and bacterial flagellin induces signaling through TLR5 (Hayashi et al., 2001). TLRs induce a range of responses including cell proliferation or maturation and the production of various cytokines, chemokines or other effector molecules, including nitric oxide and reactive oxygen intermediates (Hemmi et al., 2002; Smith et al., 2003). Signalling through TLRs leads to widespread immune induction of cellular components of the innate and adaptive immune system as well as directing the host response into particular differentiation pathways (Iqbal et al., 2005).

Cytokines are soluble gene products that are produced by different cells during the activation phase of the immune response and affect the behavior of other cells (Ferro et al., 2005). In the present study LPS was significantly ($P<0.05$) increased the mRNA expression IL-1β, IL-8 and IL-18 and non significantly the mRNA expression of TGF-β4 (Figure 2B). This indicated that lymphocytes are capable for producing inflammatory mediators following activation. Kogut et al. (2005) and Swaggerty et al. (2004) reported that the bacterial lipopolysaccharide induced and up-regulation of expression of mRNA of the pro-inflammatory cytokines IL-1β, IL-6 and IL-8 when compared to the non-stimulated controls. The

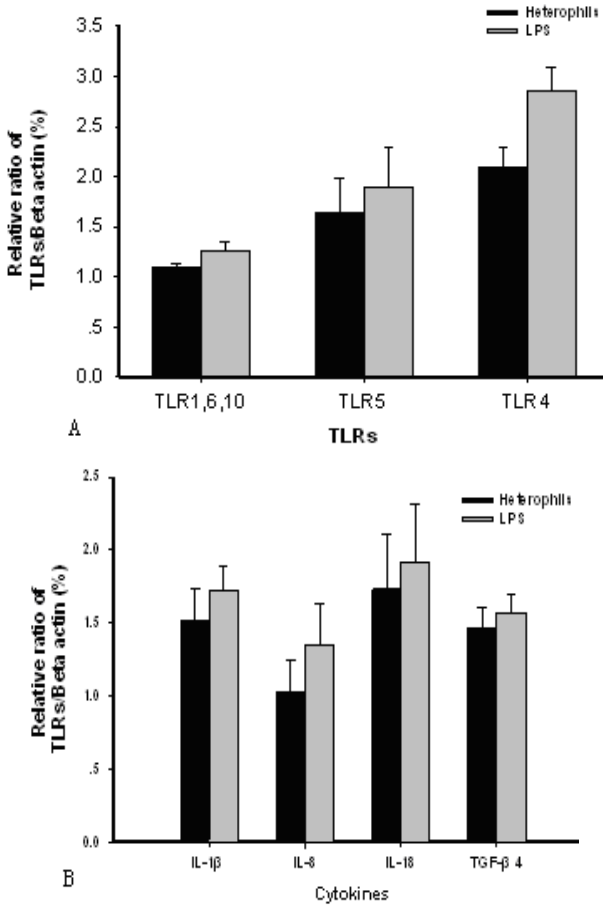

Figure 2. The mRNA expression levels of TLRs and inflammatory cytokines in avian lymphocytes induced with LPS. **A.** The relative ratio of TLRs/beta actin mRNA expression. **B.** The relative ratio of cytokines/beta actin mRNA expression. The analysis was quantitatively done by semi-quantitative RT-PCR.

increase in IL-1β, IL-8 and IL-18 mRNA expression by heterophils is associated with increased resistance to extra-intestinal SE infections in neonatal chickens and increased resistance to *Mycobacterium leprae* in leprosy patients (Swaggerty et al., 2004).

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REFERENCES

Akira S, Takeda K (2004). Toll-like receptor signalling. Nat. Rev. Immunol. 4: 499-511.
Akira S, Uematsu S, Takeuchi O (2006). Pathogen recognition and innate immunity. Cell. 124: 783-801.
Applequist SE, Wallin RP, Ljunggren HG (2002). Variable expression of Toll-like receptor in murine innate and adaptive immune cell lines. Int. Immunol. 14: 1065-1074.
Belvin MP, Anderson KV (1996). A conserved signaling pathway: the *Drosophila* Toll–Dorsal pathway. Ann. Rev. Cell Dev. Biol. 12: 393-416.
Bourke E, Bosisio D, Golay J, Polentarutti N, Mantovani A (2003). The toll-like receptor repertoire of human B lymphocytes: inducible and selective expression of TLR9 and TLR10 in normal and transformed cells. Blood 102: 956-963.
Dasari P, Nicholson I C, Hodge G, Dandie GW, Zola H (2005). Expression of toll-like receptors on B lymphocytes. Cell. Immunol. 236: 140-145
Diks SH, vanDeventer SJ, Peppelenbosch MP (2001). Lipopolysaccharide recognition, internalisation, signalling and other cellular effects. J. Endotoxin Res. 7: 335-348.
Ferro PJ, Swaggerty CL, He H, Rothwell L, Kaiser P, Kogut MH (2005). Recombinant IL-6 does not activate heterophils isolated from day-old chickens *in vitro*. Devel. Comp. Immunol. 29: 375-383.
Harris G, Kuoless R, Chen W (2006). Role of Toll-like receptors in health and diseases of gastrointestinal tract. World Gastrointestinal 12: 2149-2160.
Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, Eng JK, Akira S, Underhill DM, Aderem A (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nat. 410: 1099.
Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K, Horiuchi T, Tomizawa H, Takeda K, Akira S (2002). Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat. Immunol. 3: 196-200.
Hoebe K, Janssen E, Beutler B (2004). The interface between innate and adaptive immunity. Nat. Immunol. 5: 971-974.
Imler JL, Zheng L (2004). Biology of Toll receptors: lessons from insects and mammals. J. Leukoc. Biol. 5:18-26.
Iontcheva I, Amar S, Zawawi KH, Kantarci A, Van Dyke TE (2004). Role for Moesin in Lipopolysaccharide-Stimulated Signal Transduction. Infect. Immun. 72: 2312-2320.
Iqbal M, Philbin VJ, Smith AL (2005). Expression patterns of chicken Toll-like receptor mRNA in tissues, immune cell subsets and cell lines. Vet. Immunol. Immunopatho. 104: 117-127.
Iwasaki A, Medzhitov R (2004). Toll-like receptor control of the adaptive immune responses. Nat. Immunol. 5: 987-995.
Kogut MH, Rothwell L, Kaiser P (2005). IFN gam Priming of Chicken Heterophils Upregulates the Expression of Proinflammatory and Th1 Cytokine mRNA Following Receptor-Mediated Phagocytosis of *Salmonella enteric* serovar *enteritidis*. J. Interferon Cytok. Res. 5: 73-81.
Lee JY, Ye J, Gao Z, Youn HS, Lee WH, Zhao L, Sizemore N, Hwang DH (2003). Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids. J. Biol. Chem. 278: 37041-37051.
Smith MF, Mitchell A, Li G, Ding S, Fitzmaurice AM, Ryan K, Crowe S, Goldberg JB (2003). Toll-like receptor TLR 2 and TLR5, but not TLR4, are required for Helicobacter pylori-induced NF-kappa B activation and chemokine expression by epithelial cells. J Biol. Chem. 278: 32552-32560.
Steel RGD, Torrie JA (1980). Principles and procedures of Statistics: a Biometrical approach. McGraw Hill Book Company (second edition) Toronto. p. 481.
Swaggerty CL, Kogut MH, Ferro PJ, Rothwell L, Pevzner IY, Kaiser P (2004). Differential cytokine mRNA expression in heterophils isolated from Salmonella-resistant and -susceptible chickens. Immunol. 113: 139-148.
Takeda K, Kaisho T, Akira S (2003). Toll-like receptors. Annu. Rev. Immunol. 21: 335-376.
Takeuchi O, Kawai T, Muhiradi PF, Radolf JD, Zuchlinsky A, Takeda K (2001). Discrimination of bacterial lipopeptides by Toll-like receptor 6. Int. Immunol. 13: 933-940.
Trent M, Stead C, Tran A, Hankins J (2006). Diversity of endotoxin and its impact on pathogenesis. J. Endotoxin Res. 12: 205-223.

Verhasselt V, Buelens C, Willems F, De Grotte D, Haeffner-Cavaillon N, Goldman M (1997). Bacterial lipopolysaccharide stimulates the production of cytokines and the expression of costimulatory molecules by human peripheral blood dendritic cells: evidence for a soluble CD14-dependent pathway. J. Immunol. 158: 2919-2925.

Zarember KA, Godowski PJ (2002). Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. J. Immunol. 168: 554-561.