Toll-like receptors 2 and 4 cell surface expression reflects endotoxin tolerance in Henoch-Schönlein purpura

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SUMMARY: Canpınar H, Özaltın F, Bilginer Y, Bakkaloğlu A, Özen S. Toll-like receptors 2 and 4 cell surface expression reflects endotoxin tolerance in Henoch-Schönlein purpura. Turk J Pediatr 2010; 52: 22-27.

We aimed to analyze the Toll-like receptor (TLR)2 and TLR4 expressions, which are known to be involved in the recognition of pathogens by the innate immune system, in patients with Henoch-Schönlein purpura.

Twenty-three patients (10 males, 13 females, aged 4-16 years) with a clinical diagnosis of Henoch-Schönlein purpura were enrolled. Twenty healthy age-matched children (10 males, 10 females) served as controls. TLR2 and TLR4 expression levels on peripheral blood mononuclear cells (PBMCs) were determined by flow cytometric analysis. PBMCs were cultured with heat shock protein (HSP) 60 (1 μg/ml) as an endogenous ligand for TLR.

Levels of TLR2 and TLR4 expression on PBMC were significantly lower in the Henoch- Schönlein purpura patients compared to healthy controls when stimulated with HSP60 and with lipopolysaccharide (LPS) ($p<0.05$ for both). There was no significant difference between the stimulated and unstimulated samples from the patients.

The lower TLR response to these ligands among these patients may reflect a tolerance to bacterial antigens. Further studies will clarify whether tolerance to microbial antigens may have a role in the pathogenesis and course of Henoch-Schönlein purpura.

Key words: *Henoch-Schönlein purpura, endotoxin tolerance, heat shock protein 60, Toll-like receptor 2, Toll-like receptor 4.*

Henoch–Schönlein purpura is one of the most common vasculitides of childhood and mainly affects the vessels of the skin, gastrointestinal tract and kidneys. Clinically, it is characterized by non-thrombocytopenic purpura, arthritis/arthralgia, abdominal pain, gastrointestinal hemorrhage, and sometimes glomerulonephritis[1,2]. The specific pathogenesis is still not known. An increased CD95 expression concomitant with increased apoptosis in peripheral blood neutrophils and lymphocytes in the acute phase of Henoch-Schönlein purpura has been shown, which may play an important role in the early control of inflammatory response and repair, thereby contributing to the self-limited nature of the disease[3].

A wide variety of infections may trigger Henoch-Schönlein purpura. Group A streptococcus is the most common precipitant, demonstrable in up to one-third of cases, but exposure to *Bartonella, Haemophilus parainfluenza* and numerous vaccines and drugs may precede the development of Henoch-Schönlein purpura[4]. The role of streptococci has previously been referred to in a number of pediatric studies[5,6]. However, the role of microbial antigens in the pathogenesis of Henoch-Schönlein purpura remains elusive.

Signaling through members of the interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) family via their agonists plays an important role in immune activation, both in health and disease[7,8]. TLRs are phylogenetically conserved receptors, which have proven to be an important link between innate and adaptive immunity by

recognizing the conserved microbial protein/DNA motifs or pathogen-associated molecular patterns (PAMPs) with subsequent activation of adaptive immune responses[9-11]. Pathogen recognition through TLRs causes activation of innate immunity by inducing production of proinflammatory cytokines, such as tumor necrosis factor (TNF) alpha, IL-1, IL-18, and up-regulation of co-stimulatory molecules[12]. TLRs have been shown to be involved in the pathogenesis of several vasculitides including giant cell arteritis, retinal vasculitis in Behçet's disease and idiopathic uveitis, Kawasaki disease, as well as in the formation of atherosclerotic plaques[13-15]. Endogenous molecules such as heat shock protein 60 (HSP60) can trigger an inflammatory response via TLR2 and TLR4[16]. Because these molecules are released from stressed or necrotic cells of endogenous macromolecules, it was proposed that these TLRs might function in the biologic system as surveillance receptors that monitor tissues for disease states[17]. Since HSPs are highly conserved through evolution, one might think they can play a role in triggering of TLRs.

In the present study, we aimed to investigate whether expressions of TLR2 and TLR4 on peripheral blood cells in response to HSP60, a known endogenous ligand for TLR2 and 4, are increased in Henoch-Schönlein purpura.

Material and Methods

Study Population

Twenty-three patients (10 males, 13 females, aged 4–16 years) with a clinical diagnosis of Henoch-Schönlein purpura were enrolled in the study at Hacettepe University Pediatric Nephrology and Rheumatology Unit. All patients had active disease at the time of enrollment and blood sampling. Twenty healthy age-matched children (10 male, 10 female) served as controls. All analyses were performed in fresh blood samples in both patients and controls. The diagnosis of Henoch-Schönlein purpura was based on the recently proposed European League Against Rheumatism/Pediatric Rheumatology European Society (EULAR/PReS) criteria[2]. Antistreptolysin O (ASO) titer, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and white blood cell (WBC) count were analyzed during the acute phase of the disease. Urinalysis was performed in all, and in those with renal involvement, renal function tests and measurements of quantitative urinary protein excretion and creatinine clearance were done. TLR 2 and 4 expressions were investigated on peripheral blood cells by flow cytometry during the acute phase of the disease and before starting any therapy (see below).

In Vitro Stimulation of Peripheral Blood Mononuclear Cells

Fresh heparinized blood samples were collected from patients with Henoch-Schönlein purpura. Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized blood samples by centrifugation over Ficoll-Hypaque density gradients (Sigma, USA).

Cell Cultures and HSP60 Stimulation

Peripheral blood mononuclear cells were cultured in RPMI 1640 medium (Sigma, USA) containing 2mM L glutamine, 10% fetal bovine serum (FBS), 100 units/ml penicillin and streptomycin. Then cells were stimulated with 1 μg/ml commercially available highly purified recombinant human HSP60 (Sigma, Canada), which has been proven to have low endotoxin levels, at a density of $4x10^6$ cells in culture medium (RPMI 1640) for 4 hours at 37°C in a humidified atmosphere with 5% CO_2. All cells were cultured in 6 well plates (Nunc, Denmark). Unstimulated control cells were cultured in parallel.

Lipopolysaccharide (LPS)-Stimulated PBMCs

In a subgroup of patients and controls, LPS was used as a stimulator as a control for the HSP60 stimulation. Expression of TLR2 and TLR4 response to LPS was determined by incubation of PBMCs for 24 hours with LPS (10 ng/ml) at 37°C in a humidified atmosphere with 5% CO_2 in patients with Henoch-Schönlein purpura (n=5) and in the healthy controls (n=5).

Flow Cytometric Analysis

Fresh PBMCs ($4x10^6$cells/ml) were re-suspended in RPMI 1640 medium and 100 μl aliquots in polypropylene tubes (Becton Dickenson, Belgium). Then cells were incubated with PE conjugated TLR2 monoclonal antibody (moAb) (clone TL2, eBioscience), TLR4 moAb (clone HTA 125, eBioscience) and FITC conjugated CD14+ (clone M5E2, Becton Dickenson, Belgium) or isotypic controls moAb. After washing, cells were re-suspended in phosphate buffered saline (PBS). The

analysis was performed using two-color on flow cytometry (EPICS XL MCL Beckman Coulter, USA) with a standard argon ion laser. To identify monocytes in PBMC preparations, cells were double stained with FITC conjugated CD14. Monocytes were gated according to FS (Forward light Scatter)/ SS (Side light Scatter) plots gating, and were subsequently separated as positive cells with CD14+ staining. The analysis of TLR expression was performed using two color flow cytometry with FITC conjugated CD14 antibody and PE conjugated TLR2 and TLR4 antibodies. Relative fluorescence intensity was determined by subtracting the geometric mean fluorescence intensity of the isotype control from the sample. The expression levels of CD14, TLR2 and TLR4 were analyzed as the % fluorescence intensity (Fig. 1).

Statistical Analysis

The results were analyzed using the SPSS version 11.0 (SPSS, Inc., Chicago, IL, USA) and expressed as median interquartile range (IQR) since they showed non-normal distribution. Mann-Whitney U test was used to compare the patients and controls. Wilcoxon rank test was used to compare TLR2 and TLR4 expressions in stimulated and unstimulated samples in patients with Henoch-Schönlein purpura. A p value <0.05 was considered significant.

Results

Clinical Features

All patients enrolled in the study presented with typical purpura. In addition, 15 had arthritis/arthralgia, 4 had nephritis and 8 had gastrointestinal involvement. Nephritis was in the form of hematuria in only 2, whereas there was also mild proteinuria in the other 2. Renal functions were normal in all. The purpura subsided in a week in all of the patients. History of preceding upper respiratory tract infection was present in 65% of the patients. Serum ASO level was 243 IQR 409 IU (normal <200). WBC count, ESR and serum CRP levels were 10.100 IQR 3600/mm^3, 25 IQR 36 mm/h and 0.68 IQR 1.1 mg/dl (normal <0.5), respectively. Acute phase reactants returned to normal within a month.

Flow Cytometry

The cell surface expression of TLR2 and TLR4 on PBMCs was determined by flow cytometric analysis after culturing PBMCs in the absence or presence of HSP60. When unstimulated cells were analyzed, TLR2 and TLR4 expressions were significantly higher among patients with Henoch-Schönlein purpura when compared to controls ($p=0.001$ for both) (Table I). When stimulated with HSP60, the healthy controls had a dramatic increase in expression whereas the patients did not; the differences between the patients and controls for both TLR2 and TLR4 were significant ($p=0.002$ and $p=0.001$, respectively) (Table I).

Among the patients, stimulation with HSP60 did not increase the TLR2 or TLR4 expressions. In the patient group, TLR2 expressions in unstimulated and stimulated samples were 3 IQR 3 and 3 IQR 7, respectively ($p=0.67$), while TLR4 expressions in the unstimulated and stimulated samples were 3 IQR 2 and 1 IQR 3, respectively ($p=0.19$).

For the confirmation of HSP60 tolerance, LPS stimulation was performed in a subgroup of patients ($n=5$) and controls ($n=5$). The cell surface expression of TLR2 and TLR4 on PBMCs in response to LPS (10 ng/ml) stimulation in control subjects was again significantly higher than in patients with Henoch-Schönlein purpura (both $p=0.021$) (Table II). On the other hand, there was no significant difference in TLR expressions between unstimulated samples of the controls and patients.

Among the patients with Henoch-Schönlein purpura, the TLR2 and TLR4 expressions on mononuclear cells did not correlate with ASO, WBC, CRP, ESR, or clinical scores (data not shown).

Discussion

Henoch-Schönlein purpura is believed to be an immune mediated systemic inflammatory disease of unknown pathogenesis. TLR expression has not been evaluated in patients with Henoch-Schönlein purpura thus far. Our study showed that TLR2 and TLR4 expressions in HSP60-stimulated samples were significantly lower among Henoch-Schönlein purpura patients as compared to healthy controls. This finding was contradictory to our expectations. TLRs function as important signal transducers that mediate innate immunity and inflammatory response to pathogens through pattern recognition[17,18]. Among different TLRs, which can bind to various ligands,

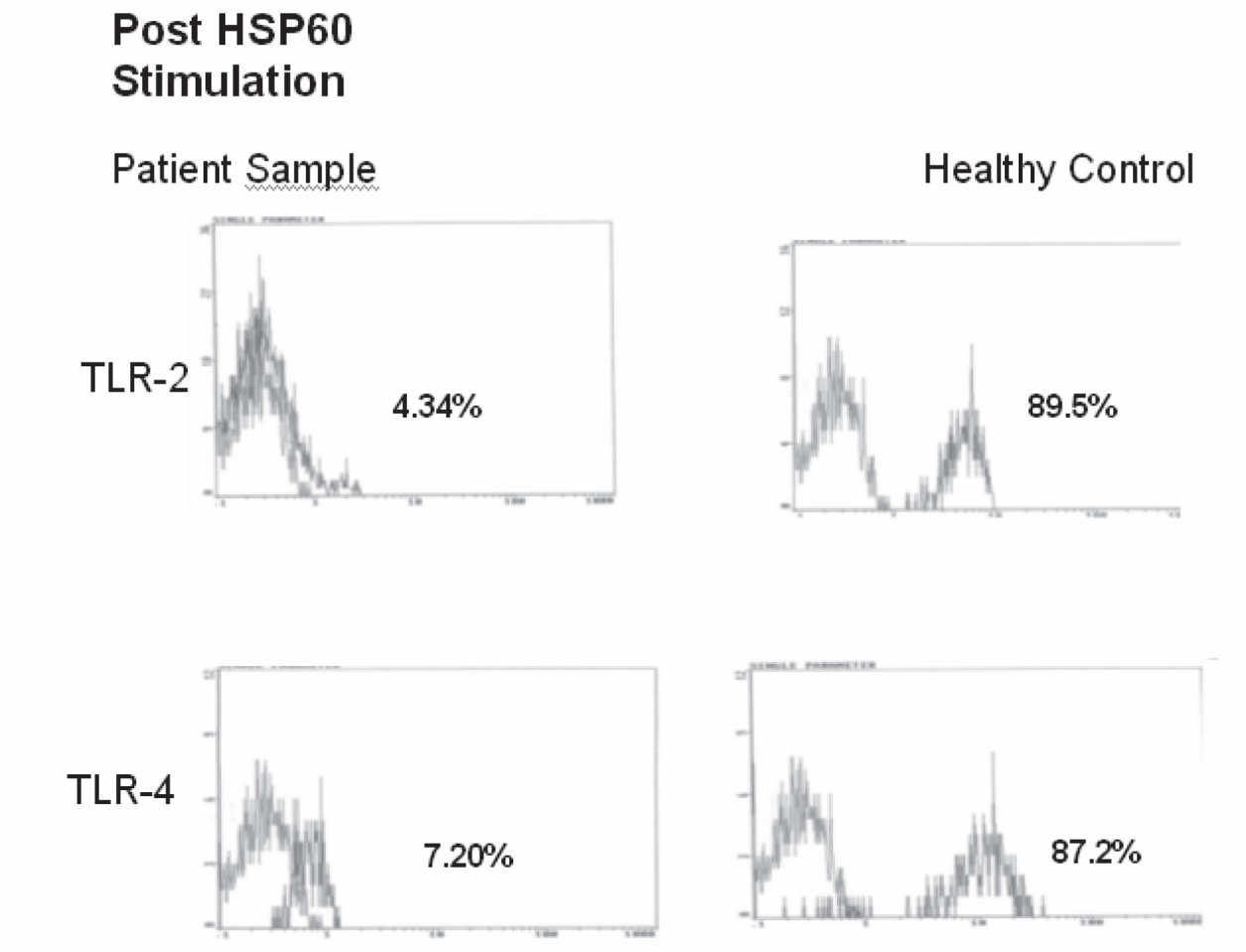

Fig. 1. TLR2 and TLR4 expressions on peripheral blood mononuclear cells in response to HSP60 (1 μg/ml, for 4 hours) in a patient with Henoch-Schönlein purpura and in a healthy control. The expression levels are indicated as mean percentage of fluorescence intensity.

Table I. Fluorescence Intensity Percent of TLR2 and TLR4 Expressions in Unstimulated and HSP60-Stimulated Cells in Patients with Henoch-Schönlein Purpura and Healthy Controls

	US-Control % (n=20)	US-Patients % (n=23)	p*	S-Control % (n=20)	S-Patients % (n=23)	p**
TLR2 Median IQR (range)	**0**	**3 IQR 3 (1-15)**	**0.001**	**91.5 IQR 7.5 (87-96)**	**3 IQR 7 (1-20)**	**0.002**
TLR4 Median IQR (range)	**0**	**3 IQR 2 (1-12)**	**0.001**	**92.5 IQR 5.2 (89-95)**	**1 IQR 3 (1-16)**	**0.001**

TLR: Toll-like receptor. IQR: Interquartile range. US: Unstimulated. S: Stimulated with HSP60. * US-control vs US-patients; ** S-control vs S-patients

Table II. Fluorescence Intensity Percent of TLR2 and TLR4 Expressions in LPS-Stimulated Cells of the Henoch-Schönlein Purpura Patients and Healthy Controls

	S-Control % **(n=5)**	S-Patients % (n=5)	p
TLR2 (Median IQR (range)	**68.5 IQR 25.5** (61-92))	30 IQR 28.5 (12-44)	0.021
TLR4 (Median IQR) (range)	**67 IQR 16** (60-78)	33.5 IQR 9.2 **(30-42)**	**0.021**

LPS: Lipopolysaccharide. S: Stimulated with LPS. TLR: Toll-like receptor. IQR: Interquartile range.

TLR4 recognizes lipopolysaccharide and gram-negative bacteria[19]. TLR4 has also been reported to be a receptor of endogenous ligands such as HSP60 and fibrinogen[16,20]. TLR4 is thought to play a crucial role in activating monocytes-macrophages in inflammatory diseases[20]. TLR2 recognizes bacterial and fungal cell wall components[21]. Although no endogenous ligand of TLR2 has been found so far, it has been reported that, in endothelial cells and macrophages, TLR2 is induced by TLR4 stimulation, thereby sensitizing cells to TLR2 ligands[22].

Heat shock protein 60 exported to the plasma membrane or released from cells is believed to be a source of danger signals, informing the innate and adaptive immune systems of tissue damage induced by various insults including injury, toxins and cellular stress, and is a scavenging molecule[23,24]. Elevated expression of HSP60 has been associated with a number of inflammatory disorders[25,26]. However, we have shown for the first time that in patients with Henoch-Schönlein purpura, expressions of TLR2 and TLR4 on HSP60- and LPS-stimulated PBMCs were significantly decreased. Down-regulation of cell surface expression of TLR 2 and TLR 4 in patients with Henoch-Schönlein purpura could be related to underlying endotoxin tolerance. Wolk et al.[27] demonstrated that human monocytes had impaired antigen presentation during endotoxin tolerance. Endotoxin tolerance is a well-known phenomenon, described both *in vivo* and *in vitro*, in which repeated exposure to endotoxin results in a diminished response, usually characterized by a reduction in proinflammatory cytokines[28]. Previous reports demonstrated the potential modulation of components of the endotoxin receptor complex during such systemic inflammation and infection as a potential mechanism for the hyporesponsiveness phenomenon[29]. A similar tolerance may have been effective in Henoch-Schönlein purpura.

The lower TLR response to HSP60 in these patients may reflect an inability to mount response to scavenging molecules. Insufficient response to stress stimuli such as that of HSP60 may have had a role in the development of endothelial injury in patients with Henoch-Schönlein purpura. Measurement of cytokine levels could be useful to better document the presented observation. Future studies will discover the implications and the nature of this tolerance in patients with Henoch-Schönlein purpura, which may have a role in the pathogenesis of the disease.

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