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Genetic Polymorphisms HLA Class II in SIRS and Sepsis in Children

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Authors' contributions

This work was carried out in collaboration between all authors. Author EE performed HLA typing, analysed data, wrote the report. Author EH was involved in collection of date and in revision of manuscript. Author JP planned the study, wrote the protocol, collected and analysed clinical data. Author IG was responsible for study planning, collection and analysing of data, she was involved in practical clinical aspects. Author LE was involved in practical clinical aspects. Authors DG and AS were involved in study planning. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: The aim of this research was to investigate the genetically determined predisposition to develop SIRS and sepsis by analyzing human leukocyte antigen HLA class II genes.
Study Design: children defined by the criteria for SIRS of Critical Care Medicine Consensus Conference were included into the study in a prospective manner.
Place and Duration of Study: Riga Stradiņš University Laboratory of Clinical Immunology and Immunogenetic, Department of Pediatrics, Rīga Stradiņš University, and Children's Clinical University Hospital, Latvia, between January 2008 and May 2009.
Methodology: Samples from children with SIRS and sepsis were collected at the Children`s Clinical University Hospital of Latvia. During the study, 65 patients with SIRS were observed. In 12 cases among SIRS patients, sepsis was confirmed. DNA was separated from lymphocytes of peripheral blood. At the same time, 100 DNA samples

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from healthy children were analysed. HLA typing low-resolution for HLA- DRB1; DQB1; DQA1 was performed by polymerase chain reaction (PCR) with amplification with sequence-specific primers (SSP). PCR products were separated on 3% arose, the amplified bands were visualised. The frequency of alleles and genotypes were compared between the patients and the controls using chi-square test. P-value and odds ratio were calculated using EPI INFO software version 6 with 95 % confidence intervals and Fisher exact correction for small numbers.

Results: The frequency of DRB1*07:01 allele was significantly increased in patients with SIRS (OR=8.71; 95% CI = 2.8-26.8; p<0.0001) and patients with sepsis (OR=9 .70; 95% CI = 2.3-42.2; p<0.005). In-group SIRS was significantly increased DQB1*03:02 (OR=2.19; 95% CI = 1.1-4.8; p<0.049) and sepsis group DQB1*03:01 (OR=2.95; 95% CI = 1.3-7.1; p<0.013) alleles also. But the frequency of DRB1*15:01 (OR=0.50; ; 95% CI = 0.2-0.9; p<0.038) and DQB1*06:01, (OR=0.16; 95% CI = 0.2-1.3; p<0.042) alleles was lower in all SIRS patients than in control group. In the distribution of HLA DRB1*/DQA1*/DQB1*, such haplotypes as DRB1*07:01/DQA1*02:01/DQB1*03:02, (OR=8.08; 95% CI = 0.9-74.2; p<0.049); DRB1*07:01/DQA1*02:01/DQB1*05:01, (OR=8.08; 95% CI = 0.9-74.2; p<0.049) and DRB1*04:01/DQA1*03:01/DQB1*02:01-2 (OR=5.10; 95% CI = 0.9-27.3; p<0.049) in SIRS group were frequently detected. The haplotypes DRB1*07:01/DQA1*02:01/DQB1*03:01, (OR=33; 95% CI = 0.9-27.3; p<0.004); DRB1*07:01/DQA1*03:01/DQB1*03:01; (OR=19.80; 95% CI = 0.9-27.3; p<0.029) and DRB1*04:01/DQA1*02:01/DQB1*03:01, (OR=19.80; 95% CI = 0.9-27.3; p<0.03) were frequently found in septic group patients.

Conclusion: Our pilot data shows that the susceptibility markers HLA-DRB1*07:01/ DQA1*02:01/ DQB1*03:01, in sepsis patients are partly consistent with literature data. The number of patients in the study is relatively small. In order to increase statistical significance of results and to prove current findings, it is important to continue the study.

Keywords: Genetic; associated genes; polymorphisms HLA-human leukocyte antigen; MHC; major histocompatibility complex; systemic inflammatory response syndrome (SIRS); sepsis.

1. INTRODUCTION

The incidence of sepsis and SIRS is still rising. Nowadays, sepsis in infants and children remains to be one of the most common problem of modern medicine. The death rate of sepsis in children is 13-50% around the world and 24,4% in Latvia [1,2]. Although technological, pharmacological, and surgical methods have improved the outcomes of many diseases, the mortality of sepsis continues to be distressingly high. Furthermore, despite the efforts in developing diagnostic tools, such as scoring systems for predicting the outcome in case of critical illnesses, and intensive care doctors are still unable to predict the outcome in all patients [3]. However, the criteria for SIRS has low specificity, and so it is necessary to find additional approaches in prognostics and diagnostics to distinguish between infectious and non-infectious aetiology [4]. Moreover, the modern level of development of genetics and molecular biology had allowed an emphasis of the important role of genetics in development of sepsis.

There is now general agreement that sepsis and the systemic inflammatory response syndrome (SIRS) are accompanied by the inability to regulate the inflammatory response. Immunological functions seem to have become dissonant, leading to progressive rampant inflammatory responses on one hand and immunosuppression on the other hand [5]. The

cause of this perturbation is still unknown. New insight into the immunopathogenesis of sepsis could promote the development of new strategies for diagnosis and therapy.

Host genetic variability in the regulatory and coding regions of gens for components of the innate immune system may influence the susceptibility to infectious diseases and can modulate diseases manifestation and outcomes [6-9]. For an efficient immune response to a pathogen to occur, HLA molecules must bind peptides derived from pathogen proteins and the T-cells repertoire must include clones that can be activated by such HLA-bound peptides [10]. Nonfulfillment of either of these requirements may render a person carrying a particular combination of HLA alleles more susceptible to given infectious diseases then one who has a different combination of alleles. The best example of this resistance is the association of specific class I and class II alleles with protection against severe malaria in sub-Saharan Africa [11]. Protection against severe malarial anaemia is afforded by procession of the HLA-B*53 and the class II HLA-DRB1*1302/DQB1*0501 haplotype.

HLA genes had been known for a long time, and the whole HLA complex had been sequenced [12]. More complex determinants of susceptibility to infection and possibly the sepsis syndrome are found in the genes located on chromosome 6 in the major histocompatibility complex (MHC). Sepsis is the result of the interaction between the microorganism and their products and the host factors released on response (cytokines and other mediators) [13]. This host response is an innate mechanism developed to protect the organism from harm but in sepsis the response is in excess, with negative effects, leading to organ dysfunction and frequently to death [14]. By altering the ability of the host to recognize a pathogenic stimulus or by altering the intensity of the inflammatory response, genetic polymorphism influences the clinical presentation and outcome of sepsis [15]. Several candidate genes have been identified as important in the inflammatory response and investigated in case-controlled studies. These HLA -DRB1; DQA1; DQB1– genes are positioned next to each other within the cluster of HLA class II genes.

In this study, we summarize the evidence for a genetic susceptibility to develop the sepsis and unfavorable outcome of sepsis. We consider the candidate genes are likely to be involved in the pathogenesis of sepsis and based on genetic variability.

The reasons why we do HLA association studies are to identify disease-specific susceptibility (risk), and to find protective markers that can be used in immunogenetic profiling, risk assessment and therapeutic decisions. HLA class II are a highly polymorphic molecule which play a pivotal role in superantigen presentation to T cells. Indeed, was founded important association between specific HLA-II haplotypes and the various manifestation of group A streptococci (GAS) sepsis [16].

To improve the diagnostics and quality of treatment in patients with sepsis, a new concept was established — systemic inflammatory response syndrome (SIRS). This syndrome, its signs and the background pathological processes have become key points in the modern concept of sepsis [17].

According to the definitions of the International Paediatric Sepsis Consensus Conference, SIRS is defined as presence of at least two of the following four criteria, one of which must be temperature or leukocyte count changes: temperature > 38.5 or < 36ºC; tachycardia (at least 2 SD above normal age group value) or bradycardia in children < 1 year old (at least 2 SD below normal age group value); respiratory rate > 2 SD above normal age group value; elevated or decreased leukocyte count according to normal age group values, or >10%

immature neutrophils. Sepsis was defined as SIRS in the presence, or as a result, of suspected or proven infection [4].

Genetic epidemiology studies suggest there is a strong genetic influence to the outcome of sepsis. The septic response is a classic example of how genetic influence modifies the response to an environmental stimulus, so called infectious insult. Francis Collins has stated that "gene isolation provides the best hope for understanding human disease at its most fundamental level" [18]. Knowledge about genetic control of cellular functions will underpin future strategies to prevent or treat disease phenotypes. The systemic inflammatory response to an infection or an injury, termed systemic inflammatory response syndrome, is likely a complex trait [19]. Most of the candidate genes hypothesised to influence the intensity of the inflammatory response are located on the highly polymorphic region of chromosome 6 known as the MHC, for humans – HLA. The ideal candidate gene for a given phenotype should have biological plausibility, and the common variant should be phenotypically neutral. Polymorphisms in HLA class II genes correlate with susceptibility to infections, including malaria, tuberculosis, leprosy, HIV, and hepatitis C and B [20-22]. Evidence of the association between HLA class II genes polymorphism and susceptibility to disease would further implicate in an adaptive immunity. In this study, we have shown genetic susceptibility to development of sepsis and unfavorable outcome of sepsis. Also we have considered the candidate genes likely to be involved in the pathogenesis of sepsis, and have discussed it potential for early prognosis of SIRS and sepsis.

In order to establish if there is any HLA association, we analysed HLA- DRB1, DQA1 and HLA- DQB1 alleles in patients with SIRS, sepsis and those of a control group.

2. MATERIALS AND METHODS

65 children (33 females, 26 males) having SIRS and admitted to the Children's Clinical University Hospital between January 2008 and May 2009, whose parents agreed to participate, were included into the study in a prospective manner. Among SIRS patients in 12 cases were confirmed sepsis. The mean (SD) age was 5.26 years. A venous blood samples were drown from each patient under local anaesthesia induced by an EMLA patch and collected at the Children`s Clinical University Hospital (Table 1).

The control group includes 100 randomly selected healthy unrelated Caucasian from the same area in Eastern Europe (65 male and 35 female, with the mean (SD) age 11.1 years) from the RSU Immunogenetic and Immunology Laboratory blood bank.

The study group of 65 children, defined by the criteria for Systemic Inflammatory Response Syndrome of Critical Care Medicine Consensus Conference. Twelve children of the SIRS group who met 3 of the following inclusion criteria of Sepsis within a 12-hour period were eligible for enrolment as sepsis patients:

1) clinical evidence of infection; 2) body temperature : hyperthermia (temperature > 38℃) or hypothermia (temperature ≤ 35.6℃); 3) heart rate > 90 beats/min); 4) respiratory rate > 20 breaths/min or arterial CO_2 tension less than 32mm Hg or a need for mechanical ventilation; 5) use of vasopressor to maintain systolic blood pressure higher than 90 mm Hg or hypotension defined as systolic blood pressure less than 90 mm Hg for more than 30 minutes or a decrease in systolic blood pressure of more than 40 mm Hg from previously established values for more than 30 minutes (hypotension had to be present at enrolment and refractory to an intravascular volume challenge of at least 500 mL); and 6) evidence of

inadequate organ function or perfusion within 12 hours of enrolment, as manifested by at least 1 of the following syndromes (previously described): acute deterioration of patients' mental status; arterial hypoxemia (PAO2/FIO2 <280); plasma lactate concentrations above the normal range or metabolic acidosis; oliguria; and disseminated intravascular coagulation [4].

Table 1. Clinical characteristics of the patients

	SIRS patients (n=65)	Sepsis patient (n=12)
Age (months)	70.4 ± 69.7[1]	97.5 ± 88.2
Number of day of symptoms at hospital admission	3.3 ± 2.5	2.9 ± 1.8
Number of days of symptoms at study entry	4.5 ± 3.0	3.8 ± 2.0
Treatment time in the hospital (days)	8.6 ± 5.9	15.4 ± 13.5
Infection focus		
Upper respiratory tract	28	-
Lower respiratory tract	19	6
Gastroenteritis	9	-
Pyelonephritis	3	-
Skin/Soft tissue infection	3	3
Osteomyelitis	1	1
Meningitis	2	2
C-reactive protein (mg/l) median	63.0	211.8

[1]*mean ± standard deviation.*

All the samples and information used in the study were coded, and patient confidentiality was preserved according to the guidelines for studies of human subjects.

The study protocol was approved by the Central Medical Ethics Committee of Latvia. Each child's parents signed a written consent form. All patients had received the standard of care according to hospital guidelines.

2.1 DNA Isolation

DNA samples were separated from proteinase-K-treated leukocytes of whole peripheral blood using the routine "salting-out" method or by phenol/chloroform extraction [23]. The DNA was stored in TE buffer. The DNA obtained was immediately used for genotyping, or it was stored at -20℃. The DNA concentration around 1 00–200 µg/ml, was determined by fluorescence with a DNA fluorimeter [24].

2.2 HLA-DR and -DQ Genotyping by PCR

HLA-DR typing for DRB1* 01:01 to 18:01 specificity, DQA1*01:01-01:03, 04:01, 06:01, and for DQB1*02:01-02:02, *03:01-03:05, *04:01-04:02, *05:01-05:04, and *06:01-06:08 was performed by PCR low-resolution using amplification with sequence-specific primers (PCR-SSP) (DNS-TECHNOLOGY, Russia). The reaction mixture (15 µl) included 1 µl DNA, 1.5 µl PCR buffer [50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, (pH 8.3)], 0.6 µl dNTPs (25 mmol/l), 1.0 µl specific primers (0.2 mmol/l), and 0.5 U of the *Taq* DNA polymerase (Promega). In addition, the internal positive control primer pair C3 and C5 was included in all

reaction mixtures at a concentration one-fifth that of the allele- and group-specific primers [25,26].

The reaction mixture was subjected to 35 amplification cycles, each consisting of denaturation at 94℃ (60 s), followed by one cycle, annealing at 94℃ (20 s), 67℃ (2 s) followed by seven cycles and extension at 93℃ (5 s), 65℃ (4 s), with a final extension in step with 28 cycles. PCR products were visualised by agarose-gel electrophoresis. After adding 2 M of loading buffer, the PCR reaction mixtures were loaded in agarose gel pre-stained with ethidium bromide (0.5 µk/ml gel). Gel was run for 15 min at 10 V/cm gel in 0.5 mM TBE buffer, then examined under UV illumination and recorded [27,28].

2.3 Statistics

Allelic frequency of HLA alleles and genotypes were compared between the patients and controls using the chi-square test. The *P* value and odds ratio (OR) were calculated using EPI INFO software, version 06, with 95% confidence intervals and Fisher exact correction for small numbers [29].

3. RESULTS AND DISCUSSION

The frequency of DRB1*07:01 allele was significantly increased in patients with SIRS (OR=8.71; 95% Cl = 2.8-26.8; p<0.0001), and in patients with sepsis (OR=9 .70; 95% Cl = 2.3-42.2; p<0.005). DQB1*03:02 (OR=2.15; 95% Cl = 1.1-4.8 p<0.049) was significantly increased in SIRS group, DQB1*03:01 (OR=2.95; 95% Cl = 1.3-7.1; p<0.013) was increased also in patients with sepsis. But the frequency of DRB1*15:01 (OR=0.50; 95% Cl = 0.2-0.9; p<0.038) and DQB1*06:01, (OR=0.16; 95% Cl = 0.2-1.3 p<0.042) alleles was lower in SIRS patients than in control subjects (Table 2).

A possible protective effect of DQA1*05:01; DQA1*01:03 was seen; the frequency of this alleles were lower in SIRS and sepsis patients, than in control group patients, but results were not statistically significant.

The most frequent HLA-DRB1/DQA1/DQB1 haplotypes in patients with SIRS were DRB1*07:01/DQA1*02:01/DQB1*03:02, (OR=8.08; 95% Cl = 0.9-74.2; p<0.05); DRB1*07:01/DQA1*02:01/ DQB1*05:01, (OR=8.08; 95% Cl = 0.9-74.2; p<0.05) and DRB1*04:01/DQA1*03:01/ DQB1*02:01-2 (OR=5.10; 95% Cl = 0.9-27.3; p<0.05). (Table 3).

The haplotypes DRB1*07:01/DQA1*02:01/DQB1*03:01, (OR=33; 95% Cl = 0.9-27.3; p<0.004); DRB1*07:01/ DQA1*03:01/DQB1*03:01; (OR=19.80; 95% Cl = 0.9-27.3; p<0.029) and DRB1*04:01/DQA1*02:01/DQB1*03:01, (OR=19.80; 95% Cl = 0.9-27.3; p<0.03) were more frequently found in patients with sepsis (Table 3).

Table 2. Frequency of DRB1*, DQA1*and DQB1* alleles in patients with SIRS and Sepsis, compared with control group patients

Group (n=65)		DRB1*07:01	DRB1*15:01	DQB1*06:01	DQB1*03:02	DQB1*03:01
SIRS n=53	gf/OR&(p)	**0.15/8.71**(0.0001)	0.22/0.50**(0.038)	0.01/0.16**(0.042)	**0.13/2.19** (0.049)	-
	CI	CI=2.8-26.8	CI = 0.2-0.9	CI = 0.2-1.3	CI = 1.1-4.8	
Sepsis n= 12	gf/OR&(p)	**0.17/9.70** (0.005)	-	-	1,09	**0.42/2.95**(0.013)
	CI	CI = 2.3-42.2				CI = 1.3-7.1
Control subjects n=100	gf	0.02	0,22	0.06	0,07	0,20

n= number of patients; gf (allele frequency),OR (odds ratio), present 95% confidence interval (CI) values and P *values were determined using two-tailed Fisher's exact test (p<0.05); Bold-face type highlights statistically significant associations for patients all groups' subjects; ** type protective statistically significant association for patients all groups' subjects*

Table 3. The frequency of DRB1*/DQA1*/ DQB1* haplotypes in patients with SIRS and sepsis, and in control groups patients

Haplotypes DRB1*/DQA1*/DQB1*		SIRS n = 53	Sepsis n = 12	Control group n=100 gf
*07:01-*02:01*03:01	gf/OR/(*p*)	-	**0.25/33/**(0.004)	0.01
	CI		CI = 3.1-350.8	
*07:01-*03:01*03:01	gf/OR/(*p*)	-	**0.17/19.80/**(0.029)	0.01
	CI		CI =1.6-238.1	
*07:01-*02:01-*03:02	gf/OR/(*p*)	**0.07/8.08/**(0.049)	-	0.01
	CI	CI = 0.9-74.2		
*07:01-*02:01*05:01	gf/OR/(*p*)	**0.07/8.08/**(0.049)	-	0.01
	CI	CI = 0.9-74.2		
*04:01-*02:01*03:01	gf/OR/(*p*)	-	**0.16/19.80/**(0.029)	0.01
	CI		CI = 1.6-238.1	
*04:01-*03:01*02:01-2	gf/OR/(*p*)	**0.09/5.10/**(0.049)	-	0.02
	CI	CI = 0.9-27.3		
*05:01-*01:03*03:01	gf/OR/(*p*)	0.04/0.24**/(0.049)	-	0.14
	CI	CI = 0.05-1.1		
*05:01-*05:01*06:01	gf/OR/(*p*)	0.19/0.26**/(0.005)	-	0.07
	CI	CI = 0.03-2.1		

n= namber of patient; gf (allele frequency), OR (odds ratio), present 95% confidence interval (CI) values and P *values were determined using two-tailed Fisher's exact test (p<0.05).; Bold-face type highlights statistically significant associations for patients all groups' subjects; ** type protective statistically significant association for patients all groups' subjects. Only statistically significant results are displayed.*

Many generations of physicians have observed and discussed the individual variability for susceptibility to infectious diseases. Why do some infants get infected with respiratory syncytial virus, and acquire respiratory failure, whilst others experience little more, than a runny nose? Why do some individuals develop a Gram-negative urinary tract infection, that responds to one dose of antibiotics, and others acquire septic shock and multiple system organ failure, despite adequate antimicrobial treatment [30-32]? Since the recognition that bacterial toxins are associated with infection, have come a long way in understanding of this condition and its pathophysiology. Recent dates increasingly realize the complexity of the septic process. Early sepsis is usually reversible, whereas patients with septic shock, often succumb despite aggressive therapy. The evidence of genetic susceptibility to common infectious diseases is growing. There is compelling evidence for genetic components, including twin studies of diseases such as tuberculosis, leprosy, malaria, and Helicobacter pylori [33]. There are > 100 examples of single-gene mutations, giving rise to severe immunodeficiency disorders [34]. A large number of genes that are known or predicted to have immunologic function reside alongside the HLA genes including HLA class II (DRB1/DQA1/DQB1); HLA-III class (heat shock protein (HSP); complement factor B; complement components 2, 4A, and 4B; TNF-a and TNF-b (lymphotoxin) [35,36]. Sepsis is the result of the interaction between the microorganisms and their products and the host factors released as a response (cytokines and other mediators). This host response is an early mechanism developed to protect the organism from the microbial attack but in sepsis the response is excessive, with negative effects leading to organ dysfunction, and frequently to death. The focus on major histocompatibility complex (MHC) class II expression came from the well demonstrated rapid (hours) and deep downregulation of HLA-DR in circulating monocytes during severe sepsis [37-39]. Specifically the HLA-II-DRB1*14/DQB1*05 (DR14/DQ5) and DRB1*07/BQB1*02 haplotypes were associated with severe forms of GAS sepsis, whereas HLA-II-DRB1*15/DQB1*06 (DR15/DQ6) haplotype conferred strong protection from streptoccocal toxic shock syndrome [40,41]. Several candidate genes had been identified as important in the inflammatory response and investigated in case-controlled studies. These HLA-DRB1/DQA1/DQB1- highly polymorphic genes are positioned next to each other within the cluster of HLA class II genes. Because these genes are associated together in linkage disequilibrium, we expected this approach would detect a haplotype-phenotype association with greater probability.

In this study, we attempted to determine, whether SIRS and sepsis are associated with any HLA class II alleles that would implicate a role for adaptive immunity in disease pathogenesis.

In the HLA class II region we found an association with HLA-DRB1*07:01, DQB1*03:01, DQB1*03:02 alleles. In such cases it can be extremely difficult to determine whether the primary, functional association is with HLA-DR or with HLA-DQ.

Certainly, HLA-DRB1 is expressed more strongly by most antigen-presenting cells, and therefore is often considered to be a functionally dominant HLA class II molecule in antigen presentation to CD4 T cells. Together with authors of “HLA and disease” could suggest that the HLA class II associations observed here could reflect simple immune response gene effects and the ability to mount a protective CD4 T cell response to key epitopes of H. influenzae, H. parainfluenzae, P. aeruginosa or S. pneumoniae [42,43].

3.1 Limitation of the Study

Although our sample size was relatively small, the result suggests that haplotype of HLA-DRB1/DQA1/DQB1 containing more than three genes was protective from immunomodulatory agents. In addition, this new approach may provide a valuable inclusion criterion for studies testing immunomodulatory agents in the treatment of SIRS/sepsis. The extension of single genomic markers to combined haplotypes including relevant alleles may be more informative and diagnostically relevant. The combination of these promising new diagnostic tools with the "finetuning" immunomodulatory capabilities of gene therapy may lead to highly focused Sepsis therapy in the future.

4. CONCLUSION

Our pilot data show that the HLA class II haplotypes DRB1/DQA1/DQB1 are associated with a higher risk of SIRS and the development of sepsis, whereas DRB1*05:01/DQA1*05:01/DQB1*06:01 are probably protective. As mentioned before, this is only suggestive, and study of a larger patient group would be required to confirm it.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

CONSENT

All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication.

ETHICAL APPROVAL

The study protocol was approved by the Central Medical Ethics Committee of Latvia. Each children parent signed a written consent form. All patients had received the standard of care according to hospital guidelines.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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