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# HLA-association of serum levels of natural antibodies

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ABSTRACT

Natural antibodies of IgM or IgG types are present in sera of most healthy individuals and are important participants of the immune response. Little is known, however, about the genetic regulation of their plasma levels in humans. We determined the concentrations of three IgM type natural autoantibodies (NAAbs) reactive to certain conserved self-antigens (citrate synthase (A-CIT), chondroitin sulphate C (A-COS) and 60 kDa heat shock proteins (A-HSP) in the sera of 78 healthy individuals and in their 86 children. In case of all the 164 individuals alleles of several polymorphisms were determined in class II (*HLA-DQ, -DR*), class III (*AGER*-429T>C, *HSP70-2* 1267A>G, *TNF*-308G>A, *CFB* S/F, copy number of the *C4A* and *C4B* genes), and class I (*HLA-A, -B*) regions of the major histocompatibility complex (MHC). Since the samples originated from a family study, extended MHC haplotypes were also determined for each study participant. Our results show that children of parents with low NAAb concentration have significantly lower serum concentrations of all the three NAAbs, as compared to offsprings of parents without reduced serum concentration. This indicates that the serum levels of these NAAbs were partly regulated by factors which are inherited from the parents to offsprings. In further studies performed only in genetically independent parents, we found significant differences in the serum levels of the IgM type A-CIT and A-COS antibodies (Abs) between carriers and non-carriers of the HLA-DR2 (15 and 16) antigens. In both cases the Ab concentrations were higher in the HLA-DR15 carriers ($p = 0.002$ and $p = 0.008$, respectively) and lower in DR16 carriers ($p = 0.029$ and $p = 0.049$, respectively) than in the non-carriers. Even more significant differences were found when the levels of two Abs were evaluated together. Frequency of the DR15 carriers was significantly lower among subjects with one or two low (in the lowest quartile) titers of A-CIT/A-COS Abs ($p = 0.014$), A-CIT/A-HSP Abs ($p = 0.016$) and A-COS/A-HSP Abs ($p = 0.013$) as compared to those with normal Ab titers for both antigens. By contrast, frequency of the DR16 carriers was significantly higher among subjects with one or two low A-CIT/A-COS Abs ($p = 0.001$), A-CIT/A-HSP Abs ($p = 0.002$) and A-COS/A-HSP Abs ($p = 0.021$) as compared to those with normal Ab titers for both antigens. Similar differences were found for both IgM type antibodies when carriers and non-carriers of the *HLA-DR15-DQ6* and *HLA-DR16-DQ5* haplotypes were considered. These novel observations indicate that not only adaptive immune response but also natural autoantibody pattern, as a part of innate immune response, is influenced by the MHC allele composition.



## 1. Introduction

Antibodies that react with self or foreign molecules detectable in the absence of known immunization with the target antigen are termed natural antibodies (Quintana and Cohen, 2004). This topic was recently reviewed by several authors in a special issue of the Journal of Autoimmunity (Avrameas et al., 2007; Cohen, 2007; Lutz, 2007; Zelenay et al., 2007). A significant part of the natural antibodies, the so-called natural autoantibodies (NAAbs) react with internal constituents of the organism, they recognize various intracellular and cell surface antigens as well as circulating macromolecules and haptens highly conserved during evolution (Avrameas et al., 2007). In humans and different animal species, these NAAbs are of IgG, IgM or IgA isotypes. NAAbs seem to be conserved during evolution suggesting that they are not simple side-products of exogenous immunization but they might have a physiological role

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in the homeostasis of the body (Cohen, 2007; Quintana and Cohen, 2004). Tissue homeostasis in vertebrates comprises the ability of the body to (a) clear proteins released by dead cells, (b) eliminate non-functional plasma proteins, (c) recognize and dispose of tumor, dead, senescent and apoptotic cells and (d) contribute to the elimination of bacteria, viruses and other pathogenic agents (Lutz, 2007). According to the "immune homunculus" theory of Irun Cohen, these antibodies have a crucial role in the control of autoimmune diseases as well (Cohen and Young, 1991).

Production of NAAbs is genetically controlled and apparently independent of environmental antigen stimulation. Several indirect data support this assumption. Even if the repertoire of NAAbs differs from one individual to another, within one individual it is stable with age (Lacroix-Desmazes et al., 1999; Mirilas et al., 1999; Mouthon et al., 1995). IgM type NAAbs can already be detected in newborn humans (Merbl et al., 2007) and animals as well as in germ-free and antigen-free mice. It seems that their repertoires are largely independent of external antigenic contacts (Haury et al., 1997). In elegant experiments Lacroix-Desmazes et al. (1999) measured immunoreactivity against different (kidney, lung, stomach and thymus) human tissue extracts in the serum samples of the same five healthy men at $43 \pm 2$ and $69 \pm 3$ years of age by using a quantitative immunoblotting technique. They found that the densitometric profiles of self-reactivity of serum IgM and of purified serum IgG remained unchanged during this 25 years interval.

Several data obtained in mice (reviewed by Zelenay et al., 2007) and recently in HIV-infected patients (Stahl et al., 2005) support the concept that similarly to antibodies against non-self-antigens, T cells contribute to the selection of natural self-antibody repertoires. Since it is well known that immune reactivity against non-self-antigens is regulated by the genes encoded in class II region of the human major histocompatibility complex (MHC), it seemed reasonable to study if the NAAbs are also controlled by these genes. Vasconcellos et al. (1998), by measuring auto-reactivity in four different MHC-congenic mice strains, demonstrated that NAAb repertoires are controlled among others by the MHC genes. Surprisingly, however, to our best knowledge, no data are available on the NAAb levels in healthy human beings carrying different class II antigens. Therefore we have decided to measure IgM type NAAbs in the sera of 78 healthy individuals with different HLA-DR and -DQ antigens. Haplotypes were determined in a family study. Levels of three different NAAbs (anti-heat shock protein 60, anti-citrate synthase and anti-chondroitine sulphate C) were measured in the serum samples of the test subjects. In order to assess the heritability of the NAAb levels we also measured the same IgM antibodies in serum samples of 86 offsprings of the test subjects. We selected these NAAbs for the study since our laboratory published several studies on the regulation of these antibodies in health and disease. IgG type antibodies reactive to the important intracellular 60 kDa heat shock protein (highly conserved in phylogeny), anti-hsp60, are known to be present in serum samples of most healthy individuals (Burian et al., 2001). We demonstrated that their level is related to the polymorphism of the *IL-6* gene in healthy Finnish blood donors (Veres et al., 2002) and healthy Hungarian individuals (Kiszel et al., 2006). We also reported that the genes of IL-6 and that encoding immunoglobulin GM show an epistatic effect on the serum concentration of anti-hsp60 (Pandey et al., 2004). In addition, in our unpublished studies (Várbíró Sz et al., submitted for publication) we found marked stability in serum levels of anti-hsp60 antibodies in healthy middle-aged persons over a 5-year-long period of time. Citrate synthase, which is present in all organisms as a key enzyme in aerobic energy metabolism, is one of the phylogenetically most conserved enzymes. This mitochondrial inner-membrane enzyme has a similar ancestry with the chloroplasts. Both have evolved by endosymbiosis from a prokaryotic cell. The plasma membrane of a prokaryotic cell is the site of oxidative phosphorylation and later on became the inner membrane of mitochondria (Nemeth et al., 1991). *In vivo* analysis of the immunological recognition of, and tolerance to this enzyme is a suitable model to investigate the mechanism of physiological and pathological autoimmune reaction. Mainly IgM isotype citrate synthase autoantibodies were found in healthy controls (Petrohai et al., 2005). Recently we summarized our studies which indicate that the anti-mitochondrial citrate synthase autoantibodies are components of the natural antibody network (Czompoly et al., 2006).

Chondroitin sulphate C is a carbohydrate antigen, it is a glycosaminoglycan attached covalently to a protein core in proteoglycans. According to our data, glycosaminoglycan-reactive IgM natural autoantibodies are highly cross-reactive with other carbohydrate structures, and are abundant in the sera of healthy adult humans, but are absent in neonates. Recently we have identified the level of natural autoantibodies to this T cell independent (TI2) carbohydrate antigen (chondroitin sulphate C) as a disease state marker in rheumatoid arthritis (B. Gyorgy et al., 2008).

Here we report on a strong association between carrier state of *HLA-DR15* and *-DR16* and the low levels of these antibodies.

## 2. Materials and methods

### 2.1. Family study

Samples were collected from healthy members of families (father, mother and at least one child) who were investigated for donor search for one of the family members. Participants were informed about the purpose of the study and they gave their informed consent for sample collection and analysis. The study was performed on 39 families involving 39 mothers, 39 fathers and 86 offspring. The number of children investigated was 1, 2, 3, 4 and 5 in 3, 28, 6, 1 and 1 families, respectively. All families included in the study were of Hungarian ethnic origin. The study was approved by the Ethical Committee of the Semmelweis University.

### 2.2. Sample collection

EDTA-anticoagulated blood was used for the preparation of genomic DNA (with commercial (Puregene) kit), while serum in which natural antibodies were determined later on was separated from native blood samples immediately after coagulation. DNA and serum samples were kept at $-30$ and $-80\,^{\circ}C$, respectively until used.

### 2.3. Determination of the MHC class II, class I and class III alleles in the DNA samples

Serologic typing for HLA-A and HLA-B was performed using the standard microlymphocytotoxicity method (Innotrain Diagnostik GmbH, Kronberg, Germany), which defined the 24 HLA-A and the 48 HLA-B antigens. *HLA-DRB1* and *HLA-DQB1* medium resolution genotyping was performed using polymerase chain reaction single-strand oligonucleotide reverse dot-blot kits (InnoLipa DRB key and InnoLipa DQB kits, respectively; Innogenetics, Zwijndrecht, Belgium). *HLA-DRB1* and *-DQB1* low-resolution typing and *DRB1*16* genotyping were performed by polymerase chain reaction with sequence-specific primers (Olerup SSP AB QIAGEN Vertriebs GmbH, Vienna, Austria). In case of the class III polymorphisms tested, *AGER*-429T>C SNP was determined by the method of Hudson et al. (2001), complement factor B (CFB) polymorphism was detected as described by Jahn et al. (1994) copy number of the *C4A* and *C4B* genes were determined as described earlier (Szilagyi et al., 2006) and *HSP70-2* 1267A>G SNP was tested by the method of Vargas-Alarcon et al. (2002). Lymphotoxin-alpha