Diabetologia (2003) 46:1100–1105
DOI 10.1007/s00125-003-1157-x

Diabetologia

The HLA-DR phenotype modulates the humoral immune response to enterovirus antigens

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Abstract

Aims/hypothesis. Enterovirus infections are among the environmental risk factors potentially contributing to the pathogenesis of Type 1 diabetes. The aim of this study was to evaluate virus-host interaction by analysing the enterovirus antibody levels in subjects carrying different HLA-DR alleles associated with either increased or decreased risk of Type 1 diabetes.
Methods. Antibodies against coxsackievirus B4 were measured to study immune responses induced by natural enterovirus infections and against poliovirus 1 to study immune responses induced by immunisation by enterovirus antigens (vaccine). Antibodies against the mumps virus were measured as a control. Study subjects included siblings of children with Type 1 diabetes taking part in the Childhood Diabetes in Finland (DiMe) Study and carrying either HLA-DR risk (DR3 and/or DR4) or protective (DR2) alleles.
Results. Children with either the HLA-DR3 or HLA-DR4 allele and those with both these risk alleles had higher Coxsackie B4 antibody levels than children carrying the HLA-DR2 allele (p=0.01, p=0.01 and p=0.008, respectively). High responders (IgG levels higher than 75 percent) were also more frequent among genetically susceptible children compared to children with the protective DR2 allele (27% vs 12%) (p<0.009). The same trend was seen for poliovirus antibodies, while mumps antibody levels had a different pattern (high responders more common among DR2-positive subjects).
Conclusions/interpretation. Diabetes-associated HLA-DR risk alleles were associated with a strong immune responsiveness and protective alleles with a weak responsiveness against enterovirus antigens. This phenomenon should be taken into consideration in serological case-control studies and it might play a role in virus-induced beta-cell damage. [Diabetologia (2003) 46:1100–1105]

Keywords HLA-DR, enterovirus, Type 1 diabetes, mumps, immune response, vaccine.

Type 1 diabetes is an immune-mediated disease resulting from the destruction of the insulin-producing beta cells in the pancreas. The pathogenesis of Type 1diabetes is still mostly unknown, but both genetic and environmental factors are important. The HLA (human leukocyte antigen) genes control immune-response functions and tissue rejection and influence susceptibility to neoplasia as well as autoimmune and infectious diseases. Certain HLA alleles are strongly associated with Type 1 diabetes and enterovirus infections are among the strongest candidates for environmental risk factors [1, 2, 3].

Enteroviruses are RNA viruses, which belong to the family of picornaviruses. They are common human pathogens causing a wide array of human diseases, including acute inflammations such as meningitis or myocarditis but mainly asymptomatic or mild

Received: 23 December 2002 / Revised: 31 March 2003
Published online: 5 July 2003
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Abbreviations: CBV4, Coxsackievirus B4; MMR, measles-mumps-rubella (vaccine); EIA, enzyme immunoassay; EIU, enzyme immunoassay unit; PV1, poliovirus 1.

respiratory infections. Enteroviral infections induce both cell-mediated and humoral immune responses, and humoral immunity plays a key role in neutralising the infectivity of extracellular viruses during viraemia.

Genetic studies have shown that patients with Type 1 diabetes are characterised by an increased frequency of the HLA-DR3 and -DR4 alleles [4] and decreased frequency of the HLA-DR2 allele [5]. The ability of HLA-DR molecules to control immune responses is reflected by the increased frequency of diabetes-associated autoantibodies in DR3- or DR4-positive subjects. In addition, a decreased frequency of T lymphocytes able to respond to mumps or coxsackievirus B4 has been observed in subjects carrying the HLA-DR3 allele and an increased frequency in subjects with the HLA-DR4 allele [6]. It has been reported that 96% of recently-diagnosed Type 1 diabetic patients positive for Coxsackie B virus-specific IgM had the HLA-DR3 and/or -DR4 allele [7]. However, the exact mechanisms by which HLA genes modulate diabetes risk are not known. Theoretically, the HLA genes could control immunisation to beta-cell autoantigens or to diabetes-inducing external agents, such as dietary proteins and viruses.

The aim of this study was to evaluate whether HLA genes control the immune response to enteroviruses by analysing enterovirus antibody levels from subjects carrying different HLA-DR alleles which are associated with either increased or decreased risk of Type 1 diabetes. We studied antibody levels against Poliovirus 1 as an indication of immune responsiveness induced by standard enterovirus exposure (polio vaccination), against Coxsackie B4 virus as an indication of immune responses induced by natural enterovirus infections and against mumps virus as an indication of immune responses to another unrelated virus.

Subjects and methods

Subjects. The Childhood Diabetes in Finland (DiMe) Study was conducted between September 1986 and April 1989 in all hospitals treating children with Type 1 diabetes in Finland. Serum samples were obtained at the diagnosis of Type 1 diabetes from a total of 780 patients and 765 siblings. The study protocol has been described in detail elsewhere [8] and it has been approved by the ethics committees of the participating hospitals. Informed consent was obtained from the families taking part in the study.

The present study series comprised siblings of affected children. Among the 765 siblings, 20 (11 boys) were homozygous for HLA-DR3, 9 (3 boys) for HLA-DR2, and 51 (19 boys) for HLA-DR4, whereas 54 (22 boys) were heterozygous for HLA-DR3/DR4, 56 (25 boys) for HLA-DR2/DRx, 70 (28 boys) for HLA-DR3/DRx, and 46 (27 boys) for HLA-DR4/DRx (x referring to alleles other than DR2, DR3 or DR4). The above 306 siblings were included in this study and their serum samples were analysed. Their mean age was 10.1 years (range 2.3–19.0). The mean ages of the seven HLA-DR subgroups did not differ.

Measles-mumps-rubella (MMR) mass vaccination was introduced in Finland in November 1982 [9]. The vaccine (Virivac, Merck, Sharp & Dohme, Rahway, N.J., USA) contains live attenuated mumps (Jeryl Lynn strain), measles (Moraten strain), and rubella (RA 27/3 strain) viruses. The first MMR vaccination is given at the age of 14 to 18 months and the second at the age of 6 years. The coverage of this vaccination has been close to 90% [10]. Accordingly most children born after 1982 have received MMR vaccine and, most likely, have not been infected naturally by mumps, measles or rubella virus. Because of this vaccination mumps is almost totally eradicated from Finland.

The Salk type of inactivated poliovirus vaccine (IPV) is given at the ages of 6 months, 1 year, 2 years and 6 years. The coverage of this vaccination has been more than 98%. In 1985 95% of the Finnish population received an extra oral polio virus vaccine dose (OPV, Sabin) because of a limited polio virus 3 epidemic in Finland. None of the children taking part in the study had a poliovirus infection that caused neurological symptoms during that epidemic.

Virus antibody analyses. IgA and IgG class serum antibodies to mumps virus were determined with a solid phase modification of an enzyme immunoassay (EIA) as described previously [11]. Purified mumps virus antigen was prepared from infected embryonated chicken eggs [12]. Harvested allantoic fluid was first centrifuged (9500 *g*, 20 min at +5°C) and the supernatant was concentrated by a hollow-fibre liquid concentrator (Amicon: Grace Company, Mass., USA). The virus concentrate was purified by centrifugation through a 30% sucrose layer on a 50% sucrose cushion (100,000 *g*, 2 h at +4°C). A visible virus band located between the sucrose layers was collected and dialysed against PBS (phosphate buffered saline) and used as antigen in EIA.

Microtitre plates (Nunc-Immunoplate, Nunc, Kamstrup, Roskilde, Denmark) were sensitised by mumps virus antigen in an optimal concentration (1 μg/ml) using overnight incubation at +4°C. Serum samples diluted 1/100 in PBS supplemented by 1% BSA, 0.05% Tween 20 and 0.5 mol/l NaCl were incubated for 60 min at +37°C. After washings, rabbit anti-human IgG (1/4000) or IgA (1/1000) (Dako Immunoglobulins, Copenhagen, Denmark) was added and allowed to react for 30 min at +37°C. The plates were again washed and alkaline phosphatase conjugated swine anti-rabbit immunoglobulin (Orion Diagnostica, Espoo, Finland) was added in a 1/100 dilution. After incubation for 2 h at +37°C 0.3 mol/l NaOH stopped the reaction and the colour reaction was recorded by a Titertek Multiskan spectrophotometer (Eflab, Helsinki, Finland). Antibody levels were expressed in enzyme immunoassay units (EIU) expressing the relative antibody activity of the sample compared with known positive and negative reference sera. Samples from at least three different HLA-DR subgroups were analysed in duplicate on one microtitre plate.

IgG and IgA class antibodies against purified coxackievirus B4 (CBV4) and purified poliovirus 1 (PV1) were measured using EIA as described [13, 14]. The purified CBV4 and PV1 were incubated at +56°C for 15 min to expose antigenic determinants common for different enterovirus serotypes.

Microtiter plates (Nunc Immunoplate, Nunc, Glostrup, Denmark) were coated by the antigens in a concentration of 1.0 μg/ml in carbonate buffer (pH 9.4). Serum samples were analysed at 1/100 (IgA) and 1/2000 (IgG) dilutions in PBS supplemented with 1% bovine serum albumin and 0.05% Tween 20. The binding of antibodies was documented using peroxidase-conjugated anti-human IgG and IgA (P214 and P216, respectively, Dako, Copenhagen, Denmark).

The results of the EIA tests were expressed in enzyme immune units (EIU), which show the relative antibody reactivity

of the sample compared to positive and negative reference sera included in each assay.

HLA typing. HLA genotypes were determined by conventional serology as described in detail earlier [15].

Statistical analysis. The Kruskall-Wallis test was used in comparisons between all HLA-DR groups. Comparisons between two HLA-DR groups were made using Mann-Whitney U test or Chi-square test. A p value of less than 0.05 was considered statistically significant.

Results

CBV4 IgG levels showed significant variation between the seven HLA-DR groups (Kruskall-Wallis test, p=0.006). Children with the HLA-DR3 allele (DR3/3, DR3/x), children with the HLA-DR4 allele (DR4/4, DR4/x) and children with both risk alleles had higher levels than children with the HLA-DR2 allele (DR2/2, -DR2/x) (p=0.01, p=0.01 and p=0.008, respectively, Fig. 1).

When homozygous and heterozygous children were analysed separately, the DR3-homozygous children had the highest antibody levels, children with HLA-DR3/4 the second highest, then children with HLA-DR4/4, HLA-DR4/x, HLA-DR3/x, while children carrying DR2/x and DR2-homozygotes had the lowest enterovirus antibody levels (Table 1).

CBV IgA levels did not show significant variation between different HLA-DR groups, but DR3-positive children tended to have higher and DR2-positive children lower antibody levels than other children (NS) (Table 1).

High responders (antibody levels higher than the 75 percentile of all samples analysed, Fig. 2) for CBV4 IgG were most frequent among DR3/DR4-heterozygous children (32%) and infrequent among children carrying the DR2 allele (DR2/2, DR2/x, 12% of the DR2-homozygous children) (p<0.02). The frequency of high responders among the children carrying the DR4 (DR4/4, DR4/x) allele was 28% (p<0.03 compared to those with DR2) and 25% among the children with the DR3 allele (p<0.04 compared to those with DR2). Overall, 27% of the children, positive for DR3 and/or DR4 were high responders compared to 12% among the children with DR2 (p<0.009).

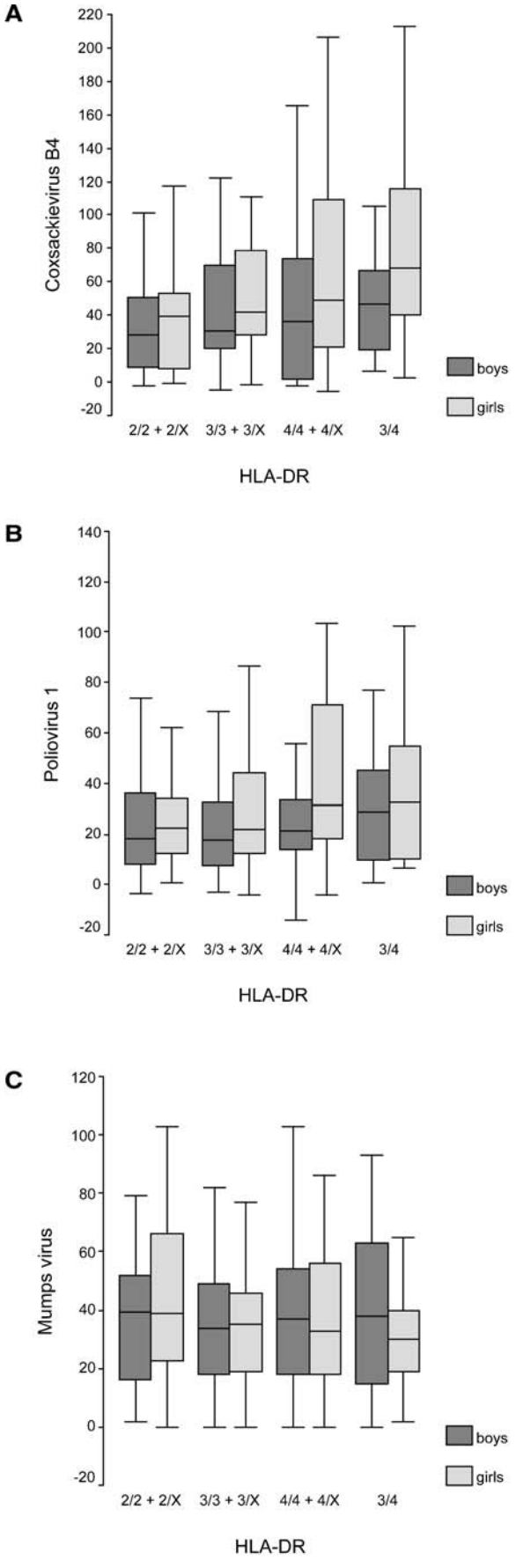

Fig. 1A–C. Coxsackievirus B4 (**A**), Poliovirus 1 (**B**) and Mumps virus (**C**) IgG levels in EIU units in four HLA-DR groups [DR2/2 + DR2/x (children homozygous or heterozygous for DR2), DR3/3 + DR3/x (children homozygous or heterozygous for DR3), DR4/4 + DR4/x (children homozygous or heterozygous for DR4) and DR3/4]. The box plots represent the median (*thick solid line*) and the 25th and 75th percentiles, while the error bars mark the lowest and highest values that are not outliers

Table 1. Virus antibody levels (mean EIU) according to various HLA-DR phenotypes

HLA-DR	N	Enteroviruses					
		Coxsackievirus B4		Poliovirus 1		Mumpsvirus	
		IgA	IgG	IgA	IgG	IgA	IgG
3/3	20	22	83	27	59	26	38
3/4	54	18	71	36	59	36	69
4/4	51	15	56	36	64	37	97
4/x	46	15	54	30	62	37	62
3/x	70	14	50	27	59	34	51
2/x	56	18	26	27	60	39	81
2/2	9	6	18	28	48	50	58

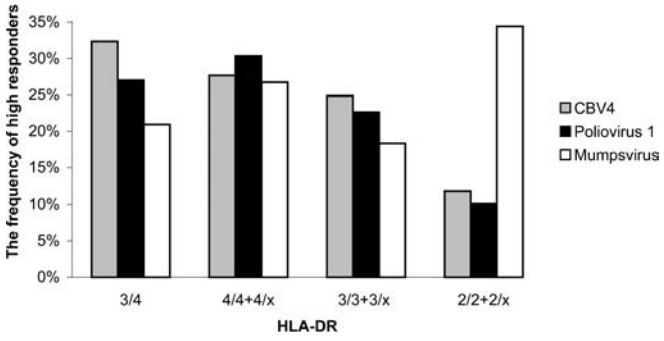

Fig. 2. Frequency of high responders (virus IgG levels over the 75 percentile) in four HLA-DR groups [DR3/4, DR4/4 + DR4/x (children homozygous or heterozygous for DR4), DR3/3+DR3/x (children homozygous or heterozygous for DR3) and DR2/2+DR2/x (children homozygous or heterozygous for DR2)]

Poliovirus 1 IgG and IgA levels did not show such a clear variation between different HLA-DR groups as CBV4 IgG (Table 1, Fig. 1B). However, high responders for PV1 IgG (Fig. 2) were most frequent among DR4-positive children (30%) and most infrequent among DR2-positive children (10%, $p<0.05$). Twenty-three percent of the DR3-positive children and 27% of the DR3/DR4-heterozygous children were high responders, respectively ($p<0.1$ and $p<0.08$ compared to DR2). Altogether 27% of the children, positive for either DR3 or DR4 were high responders compared to 10% of the DR2-positive children ($p<0.03$).

Boys had lower CBV4 IgG levels ($p=0.02$) and PV1 IgG levels ($p=0.02$) than girls. This phenomenon was seen particularly in the HLA-DR3/4 ($p=0.03$), HLA-DR3/3 ($p=0.05$) and HLA-DR4/x ($p=0.03$) groups.

High responders (Fig. 2) for mumps IgG were most prevalent among the children carrying the DR2 allele (34% of the DR2-positive children) and less common among children carrying the DR3 allele (18%) ($p<0.01$). The frequency of high responders among the DR4-positive children was 27% ($p<0.2$ compared to DR2-positive subjects) and 21% among the children with both DR3 and DR4 ($p<0.02$ compared to DR2). Mumps IgG levels did not show any apparent variation between different HLA-DR groups (Fig. 1C). They track, however, a pattern opposite to that of CBV4 IgG, being highest in DR2-positive children (Table 1). No differences were observed in the mumps IgA levels, and the levels were quite low in all seven HLA-DR groups. There was no difference between boys and girls in mumps antibody levels.

Discussion

HLA genes mediate the major part of the genetic susceptibility to Type 1 diabetes. Although the risk effect of HLA has been known for 30 years, the underlying mechanisms are still largely unknown. HLA antigens play a key role in the regulation of immune responses, and it is likely that their risk effect is somehow connected to that function. Theoretically, high-risk HLA antigens could present beta-cell autoantigens to the immune system leading to aggressive autoreactive responses, while protective HLA antigens would be unable of doing so. Alternatively, HLA could regulate the immune response against environmental triggers of Type 1 diabetes (such as dietary antigens and microbes) modulating their risk effect.

Enterovirus infections are one of the major candidates for environmental triggers of Type 1 diabetes. We analysed whether diabetes-associated HLA-DR phenotypes modulate immune responsiveness against such viruses. The results indicate that those HLA-DR alleles which mediate increased diabetes risk (DR3 and DR4) are associated with a strong humoral immune response against enterovirus antigens, while the protective HLA allele (DR2) is associated with substantially weaker immune responsiveness to enterovirus infections. This phenomenon was seen in antibodies induced by a natural enterovirus infection (antibodies against highly purified coxsackievirus B4) and also in antibodies induced by a standardised enterovirus immunisation (inactivated poliovirus vaccine), al-

though weaker. Practically all children (>98%) in Finland have been immunised with inactivated poliovirus vaccine (IPV), which induces good humoral immune responses to viral structural proteins. As a killed vaccine the vaccine virus do not replicate in the host, suggesting that the observed differences between the various HLA types are due to varying immune responsiveness to enterovirus antigens rather than a different course of infections.

This study included several subjects who were homozygous for the diabetes-associated HLA-DR3, HLA-DR4 or HLA-DR2 alleles. These homozygous subjects showed the same type of variation in antibody levels as the heterozygous patients. Children who were homozygous for DR3 or DR4 had higher antibody levels than children homozygous for DR2. Thus, there was no evidence that homozygosity as such would lead to particularly weak or strong responses. Of interest, HLA variation associated with mumps-virus antibodies was considerably lower, and, if any, showed an opposite trend compared to CBV4 antibodies with the highest levels in HLA-DR2 positive subjects. This indicates that diabetes-associated HLA-DR alleles can induce a specific effect on immune responses against enteroviruses.

The observed correlation between enterovirus specific immune responses and diabetes-associated HLA-DR alleles suggests that part of the HLA-mediated diabetes risk could be related to their role in the regulation of immune responses induced by enterovirus infections. We did not analyse T-cell responses, but it has been shown previously that HLA-DR4 is associated with a strong T-cell response to enterovirus antigens [6, 16]. This suggests that subjects carrying the HLA risk alleles for diabetes (such as HLA-DR4) develop strong responses to enterovirus antigens both in the humoral and cell-mediated arms of the immune system. It is possible that the strong immune response in subjects with the high-risk HLA alleles causes more severe tissue damage in the infected organs like pancreatic islets than the substantially weaker responses in subjects with the protective HLA-DR2 allele. Strong immune responsiveness could also lead to more severe cell damage via molecular mimicry. The possible role of HLA in enterovirus-induced beta-cell damage is supported by the fact that HLA is known to modulate the clinical course of infectious diseases (malaria [17], HIV [18], HCV [19], HBV [20], HPV [21]). For example, HLA-DR3 is associated with severe illness and HLA-B27 with a benign illness during Hantavirus infection [22]. In contrast, the weak response in HLA-DR2 positive subjects speaks against the possibility that protection against diabetes would be mediated by effective protection against diabetogenic enterovirus variants in such subjects, as protection against enterovirus infections depends mainly on antibodies.

The male sujects had lower enterovirus antibody levels than the female subjects. Reduced antibody levels in male subjects suggests that their immune response is weaker than in female subjects, and this could be connected with the higher risk of severe complications of enterovirus infections such as meningitis or carditis in male subjects than in female subjects [23]. In some areas, like in Sardinia, there is a consideraby higher incidence of Type 1 diabetes in male subjects compared with female subjects, but in many areas incidences are equal between the sexes [24].

In conclusion, our study indicates that children with HLA-DR risk alleles for Type 1 diabetes do not have a defect in their humoral immune responsiveness to enterovirus antigens which would make these children more susceptible to enterovirus infections. In contrast, the high-risk HLA-DR alleles were associated with strong immune responsiveness and protective alleles with a weak responsiveness against enterovirus antigens. This phenomenon could have pathogenetic significance e.g. by exaggerating immune pathology and cell damage in pancreatic islets in enterovirus-infected subjects who are positive for these HLA-DR alleles associated with diabetes. In addition, the study suggests that both HLA and sex are possible confounding factors in studies comparing enterovirus specific antibodies between patients with Type 1 diabetes and non-diabetic control subjects. Thus diabetic and control subjects should be matched for HLA and sex, when the role of enteroviruses is analysed in the pathogenesis of Type 1 diabetes using serological methods. This kind of matching has been done, e.g. in the prospective Finnish DIPP study [2].

Acknowledgements. This work was supported by the Sigrid Juselius Foundation, the Finnish Medical Foundation, the Academy of Finland, the Juvenile Diabetes Foundation International (grants No. 395019 and No. 197114 to H. Hyöty and grant No. 197032 to M. Knip) and the Foundation for Diabetes Research in Finland. We gratefully acknowledge the technical assistance of Ms. E. Jokela and Mrs. I. Lehtimäki.

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