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Non classical HLA genes and non-HLA genes in a population of infants at familial risk of atopy

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Abstract. *Aim:* We investigated on parental history and IgE serum level in 2588 consecutive newborns to individuate babies "at risk" of atopy at birth and we analysed the polymorphisms of class III region to evaluate the association with immunogenetic markers of HLA: C4A, C4B, LTA, RAGE and TNFA genes; we performed TNF and IgE receptor (FCERB1) physiologically related gene polymorphisms.
Result: 791 babies/2588 (30.6%) were considered "at risk" for atopy and followed-up: 400 had familial history of atopy (at least one parent or sibling), 256 had IgE $>$ 0.35 kUA/l at birth and during the follow-up and 135 were positive for both conditions. The allele C4B2 was significantly more frequent in the sample of babies at risk (22.1% vs 10%, $p < 0.001$). Furthermore, the mean value of IgE at birth in babies carrying the allele C4B2 was 2.26 KUA/l versus 0.74 KUA/l in those not carrying this allele ($p = 0.01$). No significant association emerged for RAGE at the centromeric end of class III region and for LTA, TNFA at the telomeric one. TNFRI, TNFRII and FCERB1 gene polymorphisms also seemed not implicated.
Conclusion: Our study confirms that HLA class III region seems involved in familial predisposition to atopy, and C4B gene probably acts as a marker of a more restricted subregion.

1. Introduction

The incidence, prevalence and severity of atopic disease are increasing in our industrialised countries: it seems of peculiar interest understanding the ethiology of this condition finalised to the prevention. Relationship among IgE hyperproduction and genetic predisposition or environmental influences have already been demonstrated [1]. It is well known that babies of atopic parents are at high risk of developing atopic diseases; however, the phenotypic expression of such diseases varies widely, as atopy is a heterogeneous state, probably determined by genetic and environmental interactions.

Atopic disease is often mediated by the production of immunoglobulin E (IgE) and is characterized by predominance of Th2 pathway activation. Neonates with a familial risk of IgE-mediated allergy have a significantly decreased production in cord blood of the Th1 cell secreted cytokines, IFN-γ and TNF-α, that could be correlated with atopy later in life [2,3].

Several investigators have provided evidence for a genetic susceptibility of atopy [4]. Despite evidence of heritability, it has been difficult to determine genetic markers that predispose individuals. Many studies have attempted to identify an association between this condition and immune genes and in particular with

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HLA genomic region, given its role in regulating the immune response. In particular MHC (chromosome 6p21.3) was considered to be a major locus influencing immunoglobulin levels: IgA deficit [5,6] and modulation of total serum IgE [7]. HLA class II polymorphism is variably associated with sensitisation to specific allergens, but few convincing data supporting association of HLA class II with asthma or general state of atopy, emerged [8]. Associations of asthma with HLA class III polymorphisms, in particular with Tumor Necrosis Factor (TNF), were also proposed. TNF-α, a potent pro-inflammatory cytokine, was found in excess in asthmatic airways [9,10], but these results emphasize the inflammatory nature of the asthmatic response as a distinct fact from its allergic basis [11].

In order to individuate babies "at risk" of atopy at birth we enrolled and follow-up all newborn with IgE $>$ 0.35 KU/l and/or familial history of atopy and we want to evaluate the association between these risk conditions and immunogenetic markers of HLA class III region (6p21.3): receptor advanced glycation end products (RAGE), complement serum component 4 (genes C4A and C4B), Lymphotoxin alpha (LTA), and TNFA genes. HLA class III region contains about 70 structural genes spanning 1100 Kb of genomic DNA in an unusually dense arrangement. The physiological role of many class III products are yet to be clearly determined, but many diseases (autoimmune, neurological, endocrinological and malignant) have been associated with this subregion [12–17]. Studying these polymorphisms could be useful to better understand the molecular basis of many diseases. Several genes located within HLA class III are not "immune response" genes but are critical for growth, development and differentiation as they code for receptors, extracellular matrix proteins and transcriptional factors [17–22]. Furthermore we investigated TNF and IgE receptor physiologically related genes coding for TNF receptor I (TNFRI 12p13) and II (TNFRII 1p36) and β subunit of the high-affinity IgE receptor (FCERB1 11q13) [23,24]. We presented HLA class II polymorphisms of DRB1 locus in 4 unrelated families of babies carrying the allele C4B2 and hyperIgE from birth and history of atopy, in order to better define the inheritance by descent of classical HLA predisposing genes.

2. Materials and methods

2.1. Identification and follow up of babies at risk

We investigated familial history of atopy and evaluated total serum IgE at birth in 2588 consecutive full term healthy newborns at the Division of Neonatology from January 2001 to June 2003 whose parents signed informed consent. 127 babies born in Pavia (4.7%) in the same period were not tested for lack of parental consent. Babies with parents or a sibling suffering for allergic dermatitis, rhynitis or asthma and babies with total serum IgE $>$ 0.35 KU/L were considered "at risk" and followed up: a blood sample was drawn every six months to evaluate total IgE level and specific IgE to a panel of food or inhalants allergens. The total IgE have been carried out by fluoroenzymeimmunoassay (Pharmacia CAP System FEIA. Uppsala, Sweden). IgE calibrators, traceable to the WHO preparation 75/502 for Human IgE, are used for the determination of total IgE and values are expressed in kU/l.

Atopy screening test (Phadiatop®) and multi-allergen tests (Fx5) are expressed as positive or negative using the specific IgE calibrator 0.35 kU/l as cut-off.

2.2. Genetic study design

The typing of C4 polymorphisms was planned in a subsample of 6 month-old babies scheduled for the follow-up visit in September-December 2001: thus the allele frequencies of C4A and C4B genes were evaluated in 61 Caucasian (Northern Italy) consecutive babies "at risk" who show-up at the visit out of 92 infants scheduled. At the follow-up visit, scheduled at 12 months, 35 of the 61 babies already typed for C4A and C4B at 6 months of age were typed for RAGE, LTA, TNFA, FCERB1, TNFRI and TNFRII. The remaining 26 were lost to follow-up.

To avoid Bonferroni correction for multiple tests we retested all the significant associations in an independent sample: inclusion criteria were the same of the first sampling (being at risk of atopy and being scheduled for 6 month visit in the period selected for the genetic study enrollment). 34 consecutive babies were enrolled at the 6 month visit in April-May 2002 (C4A and B typing). Sixteen babies scheduled for 6-month visit did not show-up and were not tested. 23 of these babies show up at the follow-up visit of 12 months and were typed for TNFRI in October-November 2002 (Fig. 1).

2.3. Controls

Gene frequencies of the babies at risk were compared with those of 245 blood donors of the same area (Pavia province). There were 112 females (46%), this frequency is quite similar to the one observed in our sam-

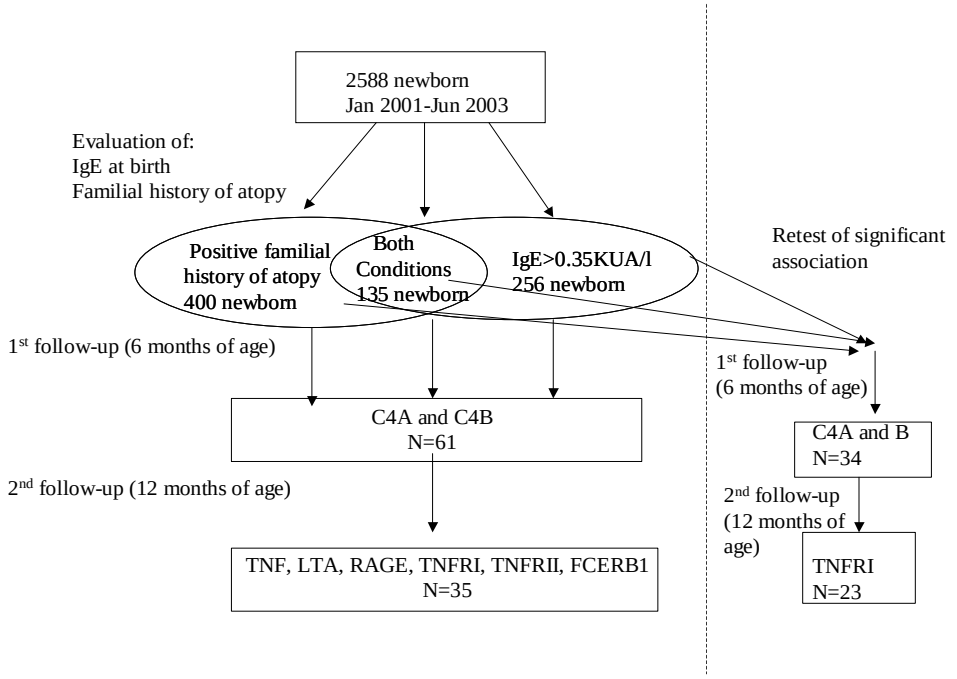

Fig. 1. Study design.

ple of infants (female: 47.3%). Mean age was 35 years, obviously older than our baby sample, but difference in genotype distribution in different age class has not been reported; so we prefer to use healthy blood donors without atopic disorders than other babies at birth. For the second sample of babies used to retest the significant associations we enrolled as controls 153 umbilical cord blood donors of our cord blood bank at the Transfusional center , born in Pavia to ensure homogeneity also with age. All babies were IgE $<$ 0.35KU/L at birth and blood samples were drawn at age of six months during routine examination.

2.4. Genetic polymorphisms

C4A and C4B polymorphisms were investigated at proteinic level. Samples of serum were conserved in EDTA, treated with carboxipeptidase and neuroaminidase and electrophoresed onto agarose gel with high voltage. The allelic proteinic bands were underlined after immunofixation with polyclonal antibodies anti-human C4 and Coomassie blue painting. The electrophoretic bands were subjected to densitometric analysis in order to define null alleles, hetero and homoduplications, as suggested by Mauff [25]. C4A and C4B genes code for two serum isophorms [12–14] and the polymorphism had been associated with many disorders [15–22].

These polymorphisms were investigated at genomic level:

LTA $+252\,G > A$: RFLP obtained by NcoI restriction according a method modified from Ozaki [26] using primer F: 5'-CCGTGCTTCGTGCTTTGGACTA-3', primer R: 5'-AGAGCTGGTGGGGACATGTCTTC-3.

RAGE $-374\,T > A$: RFLP obtained by Tsp509I restriction according a method modified from modified from Hudson [27] using the primers RAGE PROF: 5'-CCTGGGTTTAGTTGAGAATTTTTT-3' and RAGE PROR: 5'-GAAAGGCACTTCCTGGGTTCT-3'.

TNFA $-308\,G > A$: RFLP obtained by NcoI restriction according a method modified from Sakao [28] using the primers TNFRE3: 5'-AAAGTTGGGGACAC ACAA-3', TNFU2: 5'-AAATGGAGGCAATAGGTT TTGAGGGCC-3'.

TNFRI $+36\,A > G$: RFLP obtained by MspAII restriction according a method modified from Pitts [29] using primer F: 5'-GAGCCCAAATGGGGGGAGTG AGAGG-3' and primer R: 5'-ACCAGGCCCGGGCA GGAGAG-3'.

TNFRII $+196\,T > G$: RFLP obtained by NlaIII restriction according a method modified from Al-Ansari [30] using primer F: 5'-ACTCTCCTATC CTGCCTGCT-3', primer R: 5'-TTCTGGAGTTGG CTGCGTTTGT3'.

FCERB1 $-109\,C > /T$: RFLP according the method by Hizawa [31] using primer sense: 5'-

Table 1
(a) C4A and C4B polymorphism study in infants and controls

Allele	Babies "at risk"	Controls
C4A0	4 (3%)	39 (11%)
C4A2	2 (2%)	36 (10%)
C4A3	116 (95%)	260 (73%)
C4A5	0	7 (2%)
C4B0	10 (8%)	52 (15%)
C4B1	85 (70%)	269 (75%)
C4B2*	27 (22%)	35 (10%)

*$p = 0.001$.

(b) C4B2 allele distribution in different categories analysed

Allele	Babies with IgE $>$ 0.35 KUA/l	Babies with familial history of atopy	Babies with both conditions	Total Controls	
C4B2	8/38 (21.0%)[a]	14/66 (21.2%)[b]	5/18 (27.8%)[c]	27/122 (22.1%)[d]	35/356 (10%)

[a]$p = 0.05$ vs controls.
[b]$p = 0.003$ vs controls.
[c]$p = 0.03$ vs controls.
[d]$p = 0.0009$ vs controls OR $= 2.61$ (95% CI: 1.45–4.69).

GTGGGGACAATTCCAGAAGA-3' and antisense: 5'CCGAGCTGTCCAGGAATAAA-3'.

HLA-DRB1: a polymerase chain reaction sequence specific primer (PCR-SSP) technique was used to typed HLA-DRB1 at low resolution level [32].

2.5. Statistical methods

Comparisons of allele frequencies found in controls and children with IgE $>$ 0.35, children with familial history of atopy and children with both conditions were made by means of χ^2 or Fisher exact test as appropriate. The mean value of IgE at birth in babies carrying or not the allele C4B2A was compared using t-test for independent samples. A p value <0.05 was considered significant and multiple test correction was not applied since all significant association were retested in an independent sample of babies with appropriate control. All analysis were performed using STATA v 8.0 (StataCorp, College station, TX). Given the allelic frequency in the control sample and the power of the study for a true gene association of C4A and C4B alleles was 31% when OR $= 2$, 76% when OR $= 3$ and 99% when OR $= 5$.

3. Results

We enrolled 2588 consecutive babies born at Neonatology Division of IRCCS Policlinico S. Matteo in Pavia (North Italy) whose parent signed informed consent: 256 had total IgE $>$ 0.35 KU/l at birth and during the follow-up (6 months and 1 year); 400 had familial history of atopy (at least one parent or sibling), while 135 were positive for both conditions (Fig. 1). All these 791 babies/2588 (30.6%) were considered "at risk" for atopy and clinically followed-up whilst 1797 were negative both for IgE presence and familial history of atopy. The percentage of males and females in babies with IgE $>$ 0.35 KU/l was 52.7% and 47.3% respectively; among the hyperIgE infants with familiar history of atopy in 49% of cases the atopic parent was the mother, in the 41% was the father, in 8% both the parents were atopic; in the remaining 2% the familiarity was given by sibling. In nonhyperIge infants with familiar history of atopy in 35% of cases the atopic parent was the mother, in the 46% was the father, in 16% both the parents were atopic; in the remaining 3% the familiarity was given by sibling. Babies with atopic mothers are at higher risk of being hyperIgE at birth (OR 1.77 $p = 0.004$).

As shown in Table 1(a), no significant difference emerged for C4A alleles while the allele C4B2 was significantly more frequent in the sample of babies at risk (22.1% vs 10%; $p < 0.001$). As shown in Table 1(b), the frequency of the allele C4B2 is particularly high in babies that presented both hyperIgE and familial history of atopy (27.8%) while in babies with hyperIgE is 21.0%, and 21.2 in babies with familial history of atopy. All these frequencies are significantly higher than in controls ($p = 0.03$, $p = 0.05$ and $p = 0.003$ respectively). These data were confirmed in an independent

Table 2
LTA, RAGE, TNFA, TNFRI, TNFRII and FCERB1 genotypes distribution

Genotypes	Babies "at risk"	Controls	P	OR
LTA+252 AA	2/35 (5.7%)	18/241 (7.0%)		
LTA+252 AG	15/35 (42.8%)	107/241 (44.0%)		
LTA+252 GG	18/35 (51.4%)	116/241 (48.0%)		
			0.89	1.14
RAGE-374 AA	5/35 (14.3%)	49/213 (23.0%)		
RAGE-374 AT	16/35 (45.7%)	97/213 (45.5%)		
RAGE-374 TT	14/35 (40.0%)	67/213 (31.5%)		
			0.42	1.45
TNFA-308 GG	25/35 (71.4%)	185/241 (76.8%)		
TNFA-308 GA	10/35 (28.6%)	53/241 (22.0%)		
TNFA-308 AA	0	3/241 (1.2%)		
			0.57	1.41
TNFRI+36 AA	13/35 (37.1%)	74/228 (32.5%)		
TNFRI+36 AG	22/35 (62.9%)	119/228 (52.2%)		
TNFRI+36 GG	0	35/228 (15.3%)		
			0.044	infinite
TNFRII+196 MM	18/35 (51.4%)	104/190 (54.7%)		
TNFRII+196 MR	15/35 (42.9%)2	75/190 (39.5%)		
TNFRII+196 RR	2/35 (5.7%)	11/190 (5.8%)	0.93	1.07
FCERB1-109 CC	21/57 (36.8%)	38/147 (25.8%)		
FCERB1-109 CT	22/57 (38.6%)	62/147 (42.2%)		
FCERB1-109 TT	14/57 (24.6%)	47/147 (32.0%)		
			0.27*	1.44

*Subdividing babies in three categories: babies with familial history of atopy: significantly higher frequency of CC genotype (12/27, 44.4% vs 25.8% $p = 0.05$); babies with IgE > 0.35 KU/l: no significant difference was observed (5/18 27.8%I vs 27.8%) babies with both conditions: no significant difference was observed (4/12 33.3% vs 27.8%).

sample of other 34 babies (gene frequency of C4B2 in babies at "risk" 20% vs 9% in cord blood donors, $p = 0.0036$, OR 2.78, 95%CI 1.26–5.92). Furthermore, the mean value of IgE at birth in babies carrying the allele C4B2 was 2.26 KUA/l versus 0.74 KUA/l in those not carrying this allele ($p = 0.01$).

At the follow-up visit, scheduled at 12 months, a first group of these babies was typed for RAGE, LTA, TNFA and TNFRI and TNFRII. As shown in Table 2, the only significant difference is represented by TNFRI polymorphism were the genotype GG is absent in babies "at risk". To confirm our findings we retested the association in an independent sample of other 23 babies at risk presenting at the follow-up of 12 months of age. In this group the difference was no longer significant (Table 3).

Although no significant difference emerges between the whole sample of babies "at risk" and controls (Table 2), the polymorphism -10^9 in the promotor region of FCERB1 showed in babies with familial history of atopy a higher frequency of CC genotype (12/27, 44.4% vs 25.8% $p = 0.05$), while no difference was observed in babies with IgE > 0.35 KU/l and in babies with both conditions (5/18 27.8% and 4/12 33.3% respectively vs 38/147 25.8% in controls).

Table 3
TNFRI+36 G $>$ A polymorphism in an independent sample of 23 babies at risk

Genotypes	Babies at risk	Cord blood donors	p
AA	10/23 (43.5%)	51/153 (33.3%)	
AG	10/23 (43.5%)	79/153 (51.7%)	
GG	3/23 (13.0%)	23/153 (15.0%)	
			0.63

As shown in Fig. 2, investigating families, a common recurrent haplotype was not found but we noticed the alleles (inherited by the allergic parent along with C4B2) DRB1*01, *03 and *1001. These alleles were recently shown to be associated to a high serum level of IgE [33] in a white population from the Australian rural town of Busselton. The allergic parent was the mother in 3 out 4 cases; although the number of families is limited this finding could suggest to investigate a underlying parent-of-origin effect that could be in common with other immune disorders [34–37].

4. Conclusion

Identifying the genes underlying atopy status could be useful to better understand its pathogenesis and to

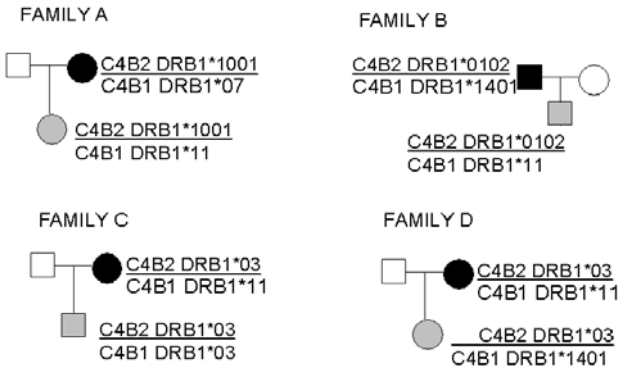

Fig. 2. Family sample. The black symbols indicate an atopic subject, the grey ones a hyper IgE infant.

improve preventive strategies and therapies. Different chromosomal regions containing genes influencing atopy have been so far analyzed and several candidate gene polymorphisms have been investigated. Our study shows that C4B gene could be involved in familial predisposition to atopy, although we cannot exclude that the high frequency of C4B2 allele could just act as a marker for other closely linked gene(s). C4B2 is not known as a functionally deficient allele; but it was associated with some autoimmune disease as uveitis [17] and diabetes [18]. C4 plays a fundamental role in antibody production as showed in hamsters, in transgenic mice experiments and in man relatively to the anti-HBV vaccine nonresponsiveness [19–22]. C4 serum protein is involved in immunocomplexes destruction, and since asthma patients were shown to have high levels of immunocomplexes a suggestive hypothesis could be that the C4B2 allele was impaired in this function. On the other hand the C4B2 allele could act simply as a marker: that is why we considered linked genes as the telomeric ones TNFA/LTA and the centromeric gene RAGE [27]. Non-HLA polymorphic genes as TNFRI and TNFRII were also analysed because functionally strictly related. No significant association was observed. Although merely speculative since the small number of families investigated, the family study could suggest that it could be interesting to investigate the HLA region between class II and III. Also regarding FCERB1 gene we did not find significant association except for babies with familial history of atopy (Table 2, footnote), differently from what reported by others: FCERB1 gene was strongly associated with asthma and hyperproduction of IgE in Japanese and Australian aborigenes [23,24].

We are following-up these babies in a precise program to evaluate a possible correlation with clinical development of overt atopic disease later in life. At present we have not children with atopic problems.

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