

Association of HLA-DQ7 antigen with cow milk protein allergy in Italian children

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In this study we investigated the HLA association with cow milk allergy. Thirty-seven Italian children with cow milk allergy and 35 randomly selected age-matched healthy children as control group were included in the study. DNA typing was performed by restriction fragment length polymorphism (RFLP) technique. We show the first statistically significant positive association between the expression of the HLA-DQ7 antigen and cow milk allergy. Several immunological tests (skin prick test, RIA, radioallergosorbent test (RAST) and ELISA) were performed to evaluate the humoral immune responses of DQ7 positive and DQ7 negative allergic patients. Our results show that among the DQ7 positive patients the majority presented a high humoral response. Furthermore, the *in vitro* proliferative response of patients to the β -lactoglobulin antigen was performed to evaluate their cell-mediated immune response. We observed that the number of the nonresponders was higher in the DQ7 positive patients when compared to the DQ7 negative patients.

Our data indicate an association of HLA-DQ7 antigen with cow milk protein allergy and that the DQ7 positive patients had a prevalence of humoral rather than cellular responses.

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An association between HLA antigens and food allergy has been found only for coeliac disease (1). A major predisposition to gluten sensitivity maps to the HLA-D region; in particular, the most important haplotype is the DQ2 that appears in 90–95% of patients (2). DQ2 is inherited in strong linkage disequilibrium with DR3 and the MHC class I antigen B8 (3). Further associations involve the HLA-DP sublocus with a prevalence of the DPB1, DPB3 and DPB4 alleles (4).

So far, many attempts have been performed to find a disease susceptibility HLA allele in other forms of food allergy, with no evidence of association. We here report the first statistically significant positive association between the expression of the HLA-DQ7 antigen and cow milk protein allergy in Italian children.

Methods

Patients

Thirty-seven children, 20 girls and 17 boys, aged between 1 month and 9 years (median age 2 years and 9 months), affected by cow milk allergy (eczema and/or gastrointestinal symptoms) were included in this study. The control group, consisting of 35 randomly selected age-matched healthy children, was used in all tests herein reported. The diagnosis of cow milk protein allergy was verified in all patients with double-blind challenge after an elimination diet period (2–4 weeks) of milk (5). The lyophilized food or placebo (talc) was given in opaque capsules, in randomized order, with a "wash-out" period of a week. The dose of lyophilized food was equivalent to 200 g of "native" food and the observation time was four hours long. When the blind challenge was negative it was confirmed by open feeding.

Immunological tests

All subjects underwent skin prick test with hydro-glycerinated allergen extracts of casein, lactalbumin

Abbreviations: RFLP: restriction fragment length polymorphism; RAST: radioallergosorbent test; PBMCs: peripheral blood mononuclear cells; PHA: phytohaemagglutinin; PCR: polymerase chain reaction; RR: relative risk; P_{cor.}: corrected p.

and β -lactoglobulin (Lofarma S.p.A., Milan, Italy), with reading after 15 minutes (6, 7). Total IgE were measured using the RIA method (Phadebas-PRIST) (8). Specific IgE were examined (Phadebas-RAST) (9) for casein, lactalbumin and β -lactoglobulin. Serum levels of cow milk casein-, lactalbumin- and β -lactoglobulin-specific IgG, IgM and IgA were measured with ELISA (10).

Proliferation assay

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Flow Laboratories, Irvine, UK) gradient separation of blood samples from 32 healthy donors and 32 cow milk protein allergic donors. Cells were then resuspended at 1×10^6 /ml in complete medium (RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum, L-glutamine and penicillin-streptomycin) and cultured in 96-well microtiter plates (Costar, Data Packaging Corp., Cambridge, MA) for 6 days in the presence of 40, 20 and 10 μ g/ml of β -lactoglobulin antigen (Sigma, St Louis, Missouri, USA) (11, 12). After 3 days phytohaemagglutinin (PHA; Famitogen, Burroughs Wellcome) (10 μ g/ml) was added to three wells as positive control of the responsiveness of PBMCs, and left throughout. Cell proliferation was measured by adding 0.5 μ Ci/well of [^{3} H]TdR (Radiochemical Inc. Amersham, UK) during the last 18 h of culture. Cells were then harvested onto glass fiber filters and [^{3} H]TdR incorporation was measured by liquid scintillation spectroscopy. Results are expressed as mean cpm $\pm$ s.d. of the mean of triplicate cultures. The average value of negative control cpm plus 3 s.d. was assumed as the threshold value for positive response.

DNA typing

DNA typing was performed by RFLP technique (13, 14). DNA was extracted from PBMCs, according to standard procedures (15). One μ g of each DNA was amplified by polymerase chain reaction (PCR) using the GH46 and GH50 primers and 2 units of *Taq* DNA polymerase (Perkin Elmer Cetus Inc.). The reaction mixture was subjected to 30 cycles of 1 min at 92°C, 1 min at 62°C, and 2 min at 72°C. Aliquots (10 μ l) of the amplified DNAs were digested with 3–5 units of *Hinf* I, *Hph* I, *Hha* I, *Kpn* I, *Fok* I and *Sac* II restriction endonucleases (Boehringer Mannheim, Indianapolis, IN) for 6 h, adding the appropriate incubation buffer and distilled H₂O up to a final volume of 15 μ l. Samples of the restriction enzyme cleaved DNAs were subjected to electrophoresis in 12% polyacrylamide gel in a minigel apparatus (Mupid, Cosmo Bio Co. Ltd).

Restriction fragments were detected by staining with ethidium bromide.

Statistical analysis

The frequency of HLA antigens was statistically analysed by the χ^2 analysis or by Fisher's exact test when the number of positivities for a particular antigen was less than five. Relative risk (RR) was then calculated and p values were corrected (P_{cor}) for the number of antigens tested. Results of immunological tests for the DQ7 positive and the DQ7 negative groups were compared using the χ^2 test with the Yates' correction.

Results

Distribution of the frequency of HLA-DR and -DQ antigens in cow milk allergic and normal children is shown in Table 1. While the expression of a particular HLA-DR antigen was not associated with the disease, the frequency of the DQ7 antigen was much higher in allergic patients than in controls (56.8% vs 22.9%, RR=4.42, $\chi^2=7.24$, $P_{cor}<0.05$).

Results of immunological tests performed on the DQ7 positive and the DQ7 negative allergic patients are shown in Fig. 1. The skin prick test (Fig. 1A) was positive in 14/21 (67%; $p<0.01$) of the DQ7 antigen positive patients and total IgE levels (Fig. 1B) were higher than the mean $\pm$ 2 s.d. in 17/21 (81%; $p<0.025$) of the same subjects. Cow milk protein-specific IgE (Fig. 1C) and IgA (Fig. 1D) were

Table 1. Distribution of the frequency of HLA-D region antigens in cow milk allergic patients and normal controls.

	HLA	Food allergy (n=37)	Control (n=35)	RR	χ^2	P_{cor}
DR	1	0 (0%)	3 (8.6%)	0		NS
	3	5 (13.5%)	5 (14.3%)	0.93		NS
	4	12 (32.4%)	8 (22.8%)	1.62		NS
	6	3 (8.1%)	1 (2.8%)	3		NS
	8	3 (8.1%)	2 (5.7%)	1.45		NS
	9	2 (5.4%)	1 (2.8%)	1.9		NS
	11	15 (40.5%)	13 (37%)	1.15		NS
	12	1 (2.7%)	1 (2.8%)	0.94		NS
	13	0 (0%)	6 (17%)	0	4.86	NS
	14	11 (29.7%)	12 (34%)	0.81		NS
	15	3 (8.1%)	4 (11.4%)	0.68		NS
DQ	2	4 (10.8%)	7 (20%)	0.48		NS
	4	2 (5.4%)	4 (11.4%)	0.44		NS
	5	19 (51.3%)	15 (42.8%)	1.4		NS
	6	5 (13.5%)	13 (37.1%)	0.26	4.17	NS
	7	21 (56.8%)	8 (22.9%)	4.42	7.24	<0.05
	8	0 (0%)	4 (11.4%)	0		NS
	9	2 (5.4%)	1 (2.8%)	1.9		NS

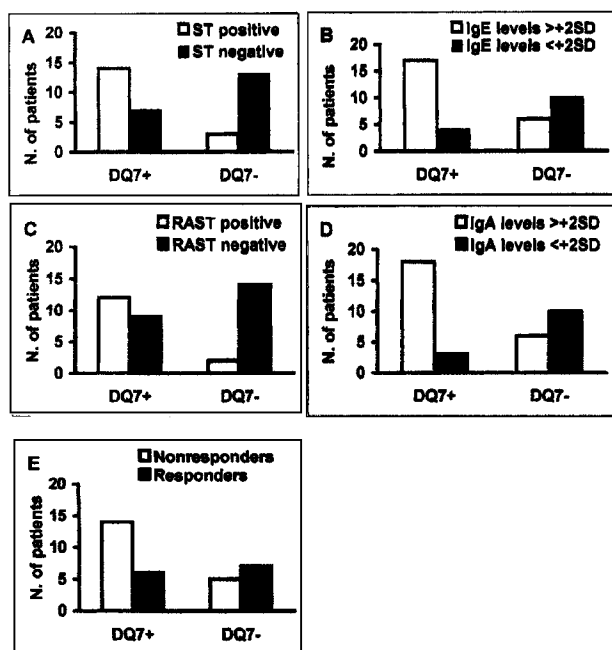


Fig. 1. Immunological tests performed on the DQ7 positive and the DQ7 negative cow milk allergic patients. (A) Skin prick test shows that 14/21 (67%) of patients who expressed the DQ7 antigen gave a positive reaction. (B) Total IgE levels were higher in the DQ7 positive patients as compared to the DQ7 negative subjects. Cow milk protein-specific IgE (C) and IgA (D) were present in 12/21 (57%) and 18/21 (86%) of the DQ7 positive children, respectively. (E) Among the DQ7 positive patients, 14/20 (70%) showed no proliferative response to the β -lactoglobulin antigen, while in the DQ7 negative patients there was a slight prevalence of the responders over the nonresponders.

present in 12/21 (57%; $p < 0.025$) and 18/21 (86%; $p < 0.01$) of the DQ7 positive patients, respectively. On the other hand, no significant differences were observed in specific IgM and IgG levels in the same patients when compared to the control group (data not shown).

The *in vitro* proliferative response of allergic patients to the β -lactoglobulin antigen was also evaluated (Fig. 1E). Interestingly, among the DQ7 positive patients, 14/20 (70%) showed no proliferative response to the β -lactoglobulin antigen, while in the DQ7 negative patients there was a slight prevalence of the responders over the nonresponders. PBMCs from the DQ7 positive and the DQ7 negative allergic patients proliferated in response to the PHA mitogen, indicating that their proliferative activity was unaffected (data not shown).

Discussion

A significant association between HLA antigens and food intolerance has been found so far only for coeliac disease. In this study we report the first evi-

dence of a statistically significant positive association of cow milk protein allergy with the HLA-DQ7 expression in Italian children.

The immunological tests (skin prick test, RIA, RAST and ELISA), performed to evaluate the humoral immune responses of cow milk allergic patients, showed that the majority of the DQ7 positive patients showed a prevalence of humoral responses. Furthermore, we analysed the *in vitro* proliferative response of the allergic patients to the β -lactoglobulin antigen to evaluate their cell-mediated immune response. Our results show that the number of the nonresponders was higher in the DQ7 positive patients when compared to the DQ7 negative patients.

In conclusion, these results suggest that the expression of the HLA-DQ7 antigen increases the risk of developing cow milk allergy in Italian children. Further experiments will be performed to assess which HLA-DQ heterodimer is involved.

The herein reported association should be included in the other examples of primary association of particular DQ alleles with various diseases, such as type I diabetes, coeliac disease, rheumatoid arthritis and multiple sclerosis (16).

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