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Normal C3b receptor (CR1) genomic polymorphism in patients with insulin-dependent diabetes mellitus (IDDM): is the low erythrocyte CR1 expression an acquired phenomenon?

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SUMMARY

Expression of the erythrocyte complement receptor (C3bR = CR1 = CD35) and its genomic polymorphism (*Hind*III RFLP) was studied in a group of 80 patients with IDDM, 31 healthy siblings and 101 healthy blood donors. Defective CR1 expression was found in 26% of the patients with IDDM compared with 9% of the controls ($P < 0.05$) and 0% of the siblings. The CR1 gene polymorphism of the IDDM patients did not significantly differ from that of the controls. The presence of a 6·9 kb (L) CR1 gene fragment was associated with a low CR1 expression in the patients ($P < 0.05$) and especially in the controls ($P < 0.001$). No significant association was found between the presence or absence of the HLA risk antigens for IDDM and CR1 expression. The results confirm that erythrocyte CR1 expression is genetically determined, but the CR1 deficiency associated with IDDM seems to be an acquired rather than a genetic phenomenon.

Keywords type I diabetes mellitus C3b receptor CR1 genomic polymorphism

INTRODUCTION

Both genetic and environmental factors contribute to the etiology of IDDM. The genetic association with HLA antigens, particularly HLA-DR3 and DR4, is well known, but non-HLA genes may also contribute to IDDM susceptibility [1]. One candidate deserving investigation in this respect is the polymorphism of the C3b receptor.

The C3b receptor or complement receptor 1 (CR1 = CD35) is expressed on several cell types, including erythrocytes [2] and most types of leucocytes [2–5]. Its biological functions include transfer of C3b-bearing immune complexes to the liver [6] and phagocytosis of C3b-opsonized particles and endocytosis of immune complexes by mononuclear phagocytes and granulocytes [7,8]. It may also be involved in the regulation of B cell differentiation and antibody production [9,10].

A defective erythrocyte CR1 has been described in autoimmune diseases, especially in systemic lupus erythematosus (SLE) [11–16]. A genetically determined CR1 deficiency could be involved in the pathogenesis of the disease, but the low expression could equally well be an acquired phenomenon caused by disease-associated immune complexes [12,14–18].

We have previously found decreased CR1 expression on erythrocytes of patients with IDDM [19]. The levels of the receptor were measured by immunoadherence haemagglutination [13], which characterizes the functional expression of CR1. The CR1 gene has been cloned [20] and it has a polymorphic *Hind*III restriction site. Low CR1 levels have been reported to concur with the presence of the 6·9 kb CR1 gene fragment in healthy persons [20,21]. In the present work we correlate the genomic polymorphism of CR1 in IDDM with functional demonstrability.

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PATIENTS AND METHODS

Patients and controls

The subjects consisted of 80 patients with IDDM from the Department of Pediatrics, Oulu University Hospital, Oulu, Finland. They were all under 15 years of age at the time of diagnosis, and 35 of them were female and 45 male. Two control groups were included, the first consisting of 101 healthy volunteer blood donors and the second of 31 healthy siblings of other patients with IDDM.

Restriction fragment length polymorphism

Peripheral blood neutrophils were isolated by a modification of the method of Boyum [22]. Erythrocytes were lysed with ammonium chloride, and DNA was obtained from the neutrophils and purified by SDS and proteinase K digestion followed by phenol and chloroform:isoamyl alcohol (24:1) extraction [23]. The DNA was then digested with *Hind*III restriction enzyme (2 U/μg DNA) at 37°C overnight and the fragments

separated on a 0·7% agarose gel (10 μg/lane) for 20 h in 0·0089 M Tris/0·0089 M borate/0·002 M EDTA, pH 8·0. The molecular mass marker was *Hind*III-digested phage lambda DNA. The fragments were transferred onto nylon membranes (Hybond-N; Amersham International, Amersham, UK) by the method of Southern [24] after denaturation. A 0·8 kb *Eco*RI insert from pBR322 plasmid related to the CR1 gene (kindly donated by Dr L. B. Klickstein, Harvard Medical School, Boston, MA) was used as the probe [25], which was labelled with a Nick Translation Kit (Boehringer Mannheim, Mannheim, Germany). The washing before prehybridization was carried out in 1% SDS/0·1 × SSC at 65°C for 1 h, and hybridization itself at 42°C for 2 h in 50% formamide, 5 × SSC, 20 mM sodium phosphate, pH 6·5, 1 × Denhardt's solution, 5% dextran sulphate and 200 μg/ml salmon sperm DNA. After hybridization the filters were washed twice in a high salt buffer (0·1% SDS/2 × SSC) for 10 min at room temperature and three times in a low salt buffer (0·1%SDS/0·1 × SSC) for 20 min at 55°C. They were then exposed for at least 36 h at −70°C using Hyperfilm-MP (Amersham) with an intensifying screen.

CR1 determinations

The immune adherence haemagglutination method (IAHA) was used for the detection of CR1 on the erythrocytes [13]. Briefly, a dilution series of heat-aggregated human IgG (25 μl/well) started from 50 μg/ml was made in U-bottomed microwells. Subsequently, 25 μl guinea pig complement at a dilution of 1:80 was pipetted into the wells and the plates were incubated for 30 min at +37°C. After adding 25 μl DTT-EDTA-veronal buffer (3 mg/ml DTT, 0·04 mol/l EDTA) to each well, 25 μl of 1% erythrocyte suspensions were pipetted into the wells. The agglutinations were read after 1 h of incubation at room temperature. If there was no agglutination, the cells were interpreted as CR1-negative. Weak reactions were recorded as positive. Each series was controlled by including known CR1-positive erythrocytes.

Statistical analysis

The χ^2-test was used for comparisons between the groups.

RESULTS

Low erythrocyte CR1 expression as measured by IAHA was more common in the IDDM patients than in the normal controls ($P < 0·05$), while none of the healthy siblings of other IDDM patients had decreased CR1 expression (Table 1). The frequencies of the allelic 7·4 kb (U) and 6·9 kb (L) fragments did not differ between the patients with IDDM and the control group, however (Table 2).

The association of CR1 expression with the CR1 gene fragments obtained by RFLP analysis was then calculated. A low level of CR1 expression correlated with the L gene (6·9 kb) fragment very significantly ($P < 0·10^6$) in the controls but not so closely in the patients with IDDM ($P < 0·05$) (Table 3).

DISCUSSION

This experiment confirms our previous observation of an increased frequency of low CR1 numbers in IDDM [19]. The

Table 1. Erythrocyte CR1 expression in patients with IDDM and controls

CR1 expression* (IAHA)	Patients ($n=31$) (%)	Siblings† ($n=31$) (%)	Controls ($n=101$) (%)
Negative	8 (26)‡	0	9 (9)
Positive	23 (74)	31	92 (91)

* CR1 expression was interpreted as negative when there was no agglutination of erythrocytes with heat-aggregated IgG and complement. Weak reactions were interpreted as positive. IAHA, immune adherence haemagglutination.

† Siblings of another IDDM series.

‡ $P < 0·05$ *vs* controls and $P < 0·01$ *vs* siblings.

Table 2. Frequencies of restriction fragment length polymorphism (RFLP) patterns of the CR1 gene (*Hind*III digestion) in patients with IDDM and healthy controls

RFLP pattern*	Patients (n=75) (%)	Controls ($n=98$) (%)
LL	7 (9)	8 (8)
UL	22 (29)	22 (22)
UU	46 (61)	68 (69)

* L and U refer to the 6·9 and 7·4 kb gene fragments, respectively.

P=NS (2 × 3 table, d.f. = 2).

Table 3. Association of low CR1 expression with the restriction fragment length polymorphism (RFLP) patterns of CR1 gene fragments (*Hind*III digestion) in controls and patients with IDDM

	*Hind*III RFLP pattern*						
	LL		UL		UU		
CR1 expression†	N	(%)	N	(%)	N	(%)	
Patients							
IAHA-negative	3	(75)	2	(29)	2	(13)	
							$> P = 0·05$
IAHA-positive	1	(25)	5	(71)	13	(87)	
Controls							
IAHA-negative	5	(63)	2	(9)	1	(1)	
							$> P < 0·001$
IAHA-positive	3	(37)	20	(91)	67	(99)	
Combined							
IAHA-negative	8	(67)	4	(14)	3	(4)	
							$> P < 0·001$
IAHA-positive	4	(33)	25	(86)	80	(96)	

* L and U refer to the 6·9 and 7·4 kb gene fragments, respectively.

† IAHA-negative patients *vs* IAHA-negative controls, P=NS. IAHA-positive patients *vs* IAHA-positive controls, P=NS (2 × 3 tables, d.f. = 2). IAHA, Immune adherence haemagglutination method.

presence of a CR1 defect in IDDM is not surprising because the disease has many features in common with the prototype autoimmune disease, SLE, including HLA-DR3 [26] and autoantibody formation [27].

There are a number of papers which confirm the association of defective erythrocyte CR1 with SLE [11,12,14–16,28,29], but the defect is not specific to SLE since there are numerous examples of other diseases associated with a low CR1 level [30–35].

We have also measured erythrocyte CR1 levels in siblings of another IDDM patient series (Table 1) without finding any increased frequency of CR1 defect. This strongly suggests that low erythrocyte CR1 numbers may be acquired in IDDM and not inherited. We have also assessed CR1 levels on erythrocytes from consecutive samples and noticed that the expression of CR1 in patients with IDDM can fluctuate between high (IAHA+) and low (IAHA−), which suggests that it is influenced by clinical factors (unpublished results).

The inheritance of a low erythrocyte CR1 level is well documented in normal subjects [15,21], while results concerning SLE are contradictory. A co-dominant mode of inheritance has been proposed [16], but findings from RFLP analysis of the CR1 gene suggest that the 6·9 kb gene fragment which is associated with low levels in controls is not increased in SLE patients or their family members [36,37]. We obtained here an increased frequency of CR1 deficiency in patients with IDDM but a normal frequency of the 6·9 kb band in RFLP analysis. This points to the influence of other immunological factors, e.g. immune complexes, on the defective expression of erythrocyte CR1 in such patients.

Table 3 also shows that the patients with UU (7·4/7·4 kb bands) or UL (7·4/6·9 kb bands) RFLP patterns had low CR1 levels more often than did the controls with the same pattern. Thus, despite the genetic element associated with a high CR1 level, some other factors must affect the expression of CR1. This can also be seen in the control group, in which three IAHA-negative subjects were genotypically intermediate or of the high receptor type. The sensitivity of the IAHA method for the enumeration of CR1 is estimated to be lower than that of antibodies or C3b dimers, which could explain some of these discrepancies. In contrast, however, some authors have concluded that IAHA is particularly efficient for detecting low numbers of CR1 [12]. The IAHA-positive reactions of three individuals with a genotypically low receptor number are difficult to accept, but the possibility of technical errors and the relative difficulty of interpreting the results may account for these cases.

In conclusion, the present observations suggest that the increased frequency of low erythrocyte CR1 function observed in IDDM cases could be acquired, since no evidence was found supporting an inherited effect on CR1 levels. We assume that an erythrocyte CR1 deficiency can occur at some time during the course of any disorder which favours immune phenomena, such as the formation of autoantibodies or immune complexes.

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