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Serological evaluation of the role of cytomegalovirus in the pathogenesis of IDDM: a prospective study

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Summary To study the possible temporal association between primary cytomegalovirus infection and the appearance of islet cell autoantibodies or the development of insulin-dependent diabetes mellitus (IDDM) cytomegalovirus antibodies were analysed from follow-up sera of 46 initially non-diabetic siblings of diabetic children who either manifested clinical IDDM (22 siblings) or turned islet cell antibody positive (24 siblings) during the prospective observation (mean follow-up time 2.9 years). Secondly, cytomegalovirus antibodies were analysed during pregnancy in 96 mothers whose child presented with IDDM before the age of 7 years and in 96 control mothers who gave birth to a non-diabetic child. Thirdly, a case-control series including 90 newly-diagnosed young children with IDDM and their 90 control subjects was analysed. No seroconversions were found in cytomegalovirus antibodies during the follow-up of the 46 siblings indicating no temporal association with islet cell antibody seroconversion or manifestation of clinical diabetes. During the follow-up 17 (37 %) siblings were constantly seronegative and 29 (63 %) seropositive for cytomegalovirus IgG and there was no difference between islet cell antibody positive and negative siblings. Cytomegalovirus IgG and IgM were not different in pregnant mothers who gave birth to a subsequently diabetic child compared to control mothers, or in newly-diagnosed diabetic children compared to control children. Cytomegalovirus IgA was higher in newly-diagnosed diabetic children than in control children ($p < 0.005$). This difference disappeared when only cytomegalovirus IgG positive individuals were analysed. No correlation was found between islet cell antibodies and cytomegalovirus antibodies in newly-diagnosed diabetic patients. The results do not support the hypothesis that primary cytomegalovirus infections could initiate the cascade leading to autoimmune destruction of the beta cells. [Diabetologia (1995) 38: 705–710]

Key words Insulin-dependent diabetes mellitus, cytomegalovirus, pathogenesis, prospective study, ICA, siblings.

Primary cytomegalovirus (CMV) infections which may manifest before birth or very early in life are presumed to be always followed by persistent or recurrent infections or both [1]. It has been suggested that CMV infections may play a role in the pathogenesis of insulin-dependent diabetes mellitus (IDDM) by inducing or promoting the autoimmune process against beta cells. This has been supported by observations that the presence of the CMV genome and islet cell antibodies (ICA) are correlated in patients with newly-diagnosed IDDM [2], and that high titres of IgG class antibody against CMV are associated with ICA in healthy siblings of diabetic children [3].

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Abbreviations: IDDM, Insulin-dependent diabetes mellitus; ICA, islet cell autoantibodies; CMV, cytomegalovirus; EIA, enzyme immunoassay; EIU, enzyme immunoassay unit.

Furthermore, immunological cross-reactivity between CMV and an islet cell 38 kDa autoantigen has been described [4]. Characteristic inclusion bodies have been observed in islets of children with fatal CMV infections providing evidence that CMV may be able to cause direct damage in human beta cells [5].

The present investigation is the first prospective study on the role of CMV in the pathogenesis of IDDM. CMV antibodies were analysed from follow-up sera taken repeatedly from originally non-diabetic siblings of diabetic children who either manifested clinical IDDM or became ICA positive during the follow-up. In addition, a series comprising pregnant mothers who gave birth to a child later manifesting IDDM as well as a case-control series of children with newly-diagnosed IDDM were analysed.

Subjects and methods

A population-based study of IDDM, called "Childhood Diabetes in Finland" (DiMe) – project included a prospective family study among non-diabetic siblings of newly-diagnosed diabetic patients (index cases) as well as access to sera taken from mothers during pregnancy with the children who contracted IDDM before the age of 7 years and matched control pregnancies. A case-control series of newly-diagnosed diabetic children younger than 7 years of age was additionally collected. The design of the study has been reported earlier in detail [6].

Subjects. The prospective family study among 3–19 year old siblings ($n = 765$) comprised blood sampling at 6-month intervals immediately after the diagnosis of the index case. Of these siblings 22 presented with IDDM and 24 became ICA positive during the follow-up. The follow-up varied from 27 days to 6.3 years (mean 2.9 years) and in 33 cases (72 %) the follow-up time was more than 2 years (Fig. 1). The series of pregnant mothers comprised 96 mothers whose child presented with IDDM before the age of 7 years and 96 control mothers with a non-diabetic child matched for the time of delivery (± 1 day) and the sex of the child. The maternal sera were obtained from the National Public Health Institute and were taken at the end of the third month of pregnancy originally for the routine rubella screening. The case-control study among young-onset cases with IDDM included 90 newly-diagnosed diabetic children under 7 years of age (39 children were born to the above-mentioned mothers) and their 90 unrelated age and sex matched control subjects (39 children were born to the above-mentioned control mothers). The mean age was 4.5 years and the number of male pairs was 48 (53 %). Sera were obtained at the diagnosis of clinical IDDM (on average within 7 days in patients and 59 days in control subjects) from all patients and control children and a couple of months later from 38 patient-control pairs (on average within 78 days in patients and 123 days in control children). In 11 of the 90 case-control pairs clinical IDDM was diagnosed before the age of 2 years. An additional 21 children with IDDM before the age of 2 years were also analysed.

Cytomegalovirus antibody enzyme immunoassay. Cytomegalovirus IgG and IgM were analysed using a commercial enzyme immunoassay (EIA) test (CMV IgG/IgM EIA, Labsystems, Helsinki, Finland). CMV IgA was analysed by peroxidase-conjugated anti-human IgA (Dako Immunoglobulins, Copenhagen, Denmark) as the second antibody layer. Antibody levels were expressed in enzyme immunoassay units (EIU) indicating the relative antibody activity of the sample compared to known positive and negative reference sera [7]. The cut-off limit for IgG positivity was 15 EIU, for IgM 40 EIU and for IgA 15 EIU as deduced from the distribution of IgG, IgM and IgA antibody levels in known positive and negative sera. All follow-up sera from one subject were analysed in one microtitre plate. Coded sera from mothers of a diabetic child and from the control mothers were similarly analysed in one microtitre plate as were the sera from newly-diagnosed diabetic children and corresponding control subjects. IgG seroconversions (*de novo* appearance of CMV IgG) were taken as an indicator of primary CMV infections. An additional IgM determination was done if a CMV IgG positive individual became ICA positive (*de novo* appearance of ICA) in the very beginning of the follow-up. In pregnant mothers the identification of recent pri-

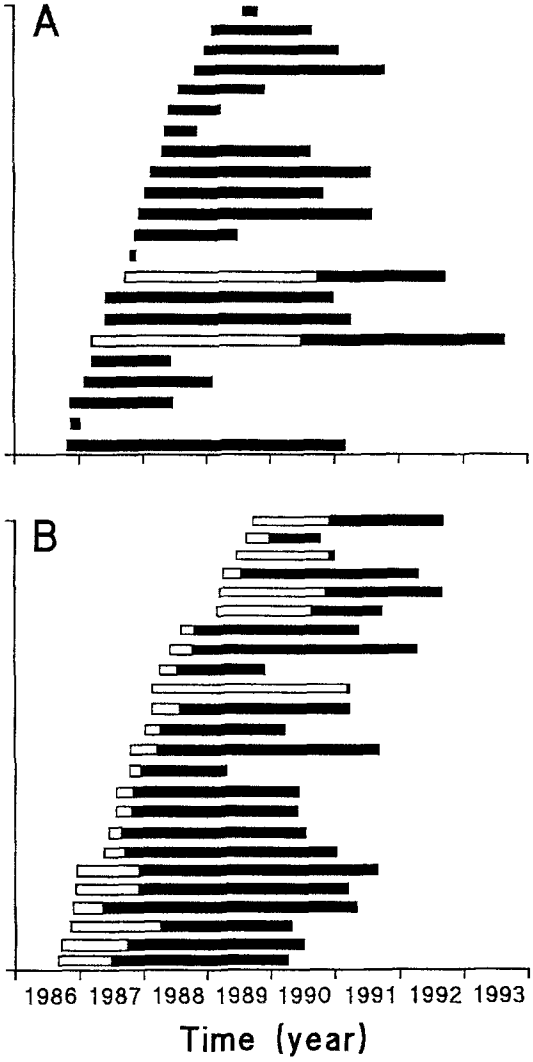

Fig. 1. (**A**, **B**) Duration and time of follow-up in 46 originally non-diabetic siblings of diabetic children: (**A**) initially non-diabetic siblings who subsequently manifested IDDM (11 males and 11 females, mean age 7.7 years at the beginning of the follow-up); (**B**) non-diabetic siblings who turned ICA positive (14 males and 10 females, mean age 9.7 years at the beginning of the follow-up). Blank box, time before ICA seroconversion; solid box, time after ICA seroconversion

mary CMV infections was done by testing a single serum sample for CMV IgM and IgG [8]. In the case of IgM positivity avidity of CMV IgG antibodies was additionally determined; low avidity (0–20 %) is characteristic of a current primary CMV infection, borderline avidity (21–35 %) refers to a primary CMV infection during the past few months, and avidity over 35 % to no primary CMV infection within the preceding 1–2 months [9].

Assay of ICA. Conventional ICA were determined by a standard indirect immunofluorescence test [10] and expressed in Juvenile Diabetes Foundation (JDF) units. The detection limit was 2.5 JDF units. Our ICA laboratory has participated in the international workshops on standardization of the ICA assay in which the sensitivity was 100 %, specificity 98 %, validity 98 % and consistency 98 % in the fourth round.

Statistical analysis

Two-tailed paired Student's *t*-test and McNemar's test were used in the statistical analyses of paired data. Correlation coefficients were calculated according to Spearman's method. As the distribution of CMV antibodies and ICAs were strongly skewed, ranks were used in the analyses of multiple regression. The analyses were done using SAS statistical software. The results are given as mean ± SEM if not otherwise indicated.

Results

Of the 46 prospectively followed siblings 29 (63 %) were originally seropositive and 17 (37 %) seronegative for CMV IgG and no seroconversions were seen during the observation period (Table 1). Twenty-two siblings manifested IDDM during the follow-up (mean follow-up 2.5 years). Half of them ($n = 11$) were seronegative for CMV IgG and remained that way until 2 months after the diagnosis of diabetes. The remaining 11 (50 %) siblings were constantly seropositive for CMV IgG showing no significant alterations in antibody levels. Twenty of those 22 pre-diabetic siblings were initially ICA positive and two were ICA negative but both became ICA positive during the follow-up. In one of them CMV IgG was constantly positive until the diagnosis of diabetes (altogether 5.1 years) and in the other constantly negative for 6.3 years. One of the 20 initially ICA-positive pre-diabetic siblings turned ICA negative before the manifestations of IDDM. Out of the 24 non-diabetic siblings who turned ICA positive, but did not present with diabetes, six (25 %) were CMV IgG seronegative and 18 (75 %) seropositive showing no seroconversions or significant alterations in CMV IgG levels during the follow-up. In 11 (46 %) of these siblings ICA positivity was only transient. None was CMV IgM positive in the subgroup of four CMV IgG positive siblings who ICA seroconverted in the very beginning of the follow-up (within 4 months). CMV IgA remained at constant levels showing no increases in association with ICA seroconversion or the diagnosis of diabetes.

Table 1. The number of ICA positive and negative siblings at the start and at the end of the follow-up period according to CMV IgG antibody status[a]

	CMV IgG		
	Positive	Negative	Total
Siblings who manifested IDDM			
ICA negative at start *(n)*	1	1	2
ICA positive at start *(n)*	10	10	20
ICA negative at end *(n)*	1	0	1
ICA positive at end *(n)*	10	11	21
Siblings who turned ICA positive			
ICA negative at start *(n)*	18	6	24
ICA positive at start *(n)*	0	0	0
ICA negative at end *(n)*	9	2	11
ICA positive at end *(n)*	9	4	13

[a] ICA positivity did not persist to the end of the observation period in one sibling who manifested IDDM and in 11 siblings who ICA seroconverted

In the series of pregnant mothers whose child contracted IDDM before the age of 7 years, 71 of 96 (74 %) had CMV IgG antibodies at the end of the first trimester of the pregnancy compared to 78 of 96 (81 %) of control mothers (NS). CMV IgG levels were also not statistically different between the two groups (104 ± 7 and 112 ± 7 EIU, respectively). Low levels of CMV IgM were found in three mothers with a diabetic child (40 EIU, 47 EIU, 64 EIU) and two control mothers (78 EIU, 42 EIU) who were all further analysed for the avidity of CMV IgG. Two mothers with a diabetic child and two control mothers had CMV IgG antibodies of high avidity (over 35 %) and one mother with a diabetic child had a borderline avidity of 35 %.

Among newly-diagnosed diabetic children CMV IgG was found in 42 of 90 (47 %) cases and in the matched control children in 38 of 90 (42 %) cases (NS). Only one of the patients had seroconversion in CMV IgG between the first and second serum sample. Four patients (4 %) and four control children were positive for CMV IgM (NS). The levels of CMV IgG or IgM were not different between the patients and control subjects (Table 2). The proportion of CMV IgA positive patients was 39 % (35 of 90) which was significantly higher ($p < 0.005$) than that in control children (16 %, 14 of 90) and also the mean level of IgA was significantly higher in patients than in control children (Table 2). This difference in IgA antibodies was more pronounced in females (16 ± 2 vs 9 ± 1 EIU, $p < 0.005$) than in males (14 ± 2 vs 10 ± 2 EIU, NS). CMV IgA was not, however, different when only IgG positive patients were compared to their IgG positive control subjects (17 pairs, 13 ± 2 vs 12 ± 2 EIU). In 14 of 90 (16 %) diabetic pa-

Table 2. CMV IgG, IgM and IgA in three different age groups in patients with newly-diagnosed IDDM and in age- and sex-matched control children

Age groups (years)								
	< 2		2–3		4–6		Total	
Status	Patients	Control subjects	Patients	Control subjects	Patients	Control subjects	Patients	Control subjects
(*n*)	11	11	22	22	57	57	90	90
IgG (EIU)	17 ± 11	42 ± 14	48 ± 11	31 ± 11	44 ± 6	42 ± 6	42 ± 5	39 ± 5
IgM (EIU)	4 ± 1	9 ± 2	14 ± 3	14 ± 3	18 ± 2	15 ± 2	15 ± 1	14 ± 1
IgA (EIU)	12 ± 5	13 ± 6	18 ± 4[a]	8 ± 1	14 ± 1[b]	9 ± 1	15 ± 1[c]	9 ± 1

Results are give as mean ± SEM.
[a] $p < 0.05$ vs control subjects; [b] $p < 0.005$ vs control subjects; [c] $p < 0.005$ vs control subjects

tients and in 2 of 90 (2 %) control subjects CMV IgA was positive but CMV IgG was negative (Fig. 2). No correlation was found between CMV IgA and age. The levels of ICA and CMV IgG, IgM or IgA did not correlate at the diagnosis of diabetes in the patients, and the mean ICA levels did not differ between CMV IgG positive and negative patients (210 ± 7 vs 204 ± 60 JDF units). This was true also when the effect of age on ICA positivity was excluded in the analysis of multiple regression. All control children were ICA negative.

Diabetic children who were diagnosed very young (before the age of 2 years, the youngest being 0.8 years of age) were analysed in more detail to ascertain possible signs of CMV infection in early life. Of these 32 patients 10 (31 %) were positive for CMV IgG but none for CMV IgM. ICA was analysed in all of these 32 patients. The majority ($n = 30$) were ICA positive including 10 CMV IgG positive and 20 CMV IgG negative patients, and the remaining two ICA negative patients were also CMV IgG negative. The levels of ICA did not correlate with CMV IgG, IgM or IgA levels. For 11 of these 32 patients an age- and sex matched control subject was available (Table 2). All these 11 pairs were CMV IgM negative. In five pairs both the diabetic patient and the control subject were IgG negative and in two pairs IgG positive, respectively. The remaining four pairs were discordant for CMV IgG; all diabetic patients were IgG negative and the control subjects IgG positive.

Discussion

The purpose of this study was to prospectively investigate the number of primary CMV infections before the onset of clinical IDDM and the possible temporal association between CMV infections and the appearance of ICA. Serological methods used in the present study have previously been shown to be useful in the diagnosis of primary but also reactivated CMV infections and CMV IgG, IgM and IgA antibodies have all been detected in primary, convalescent and recurrent infections [11]. IgG antibodies normally peak during the first month or two after the onset of infection [12] and IgM during the early course of infection [8]. In recurrent CMV infections IgG usually rises while IgM is rarely detectable [11]. CMV IgA has been found in sera of both acute and convalescent patients and it has been considered to be a marker of CMV reactivation when IgM is absent [11].

The role of CMV in the pathogenesis of IDDM has been proposed in some earlier reports [2–5, 13]. However, such a connection has not been found in all studies, for example in two separate reports there was no evidence of recent CMV infection in patients with newly-diagnosed IDDM [14, 15]. Also, in the present study, CMV IgG and IgM were not different in newly-diagnosed diabetic children and control subjects, and the proportion of seronegative patients was relatively high (53 %). This suggests that CMV is not a common precipitator of clinical IDDM. However, CMV IgA was higher in these patients compared to control children. This could reflect reactivated or persistent CMV infections in newly-diagnosed diabetic patients. On the other hand, IgA was not different in IgG positive patients and control subjects and it did not rise before the diagnosis of diabetes or before ICA seroconversions during the follow-up of initially unaffected siblings of IDDM patients. Also the observation that CMV IgG was not different in diabetic patients and control subjects suggests an equal exposure to CMV in the past and argues against an over-representation of patients with chronic forms of CMV infections. Thus, higher CMV IgA in diabetic patients may reflect non-specific IgA reactivity in these children.

Comparison of antibodies between patients and control subjects at the time of the diagnosis of diabetes has some disadvantages. The initial virus infec-

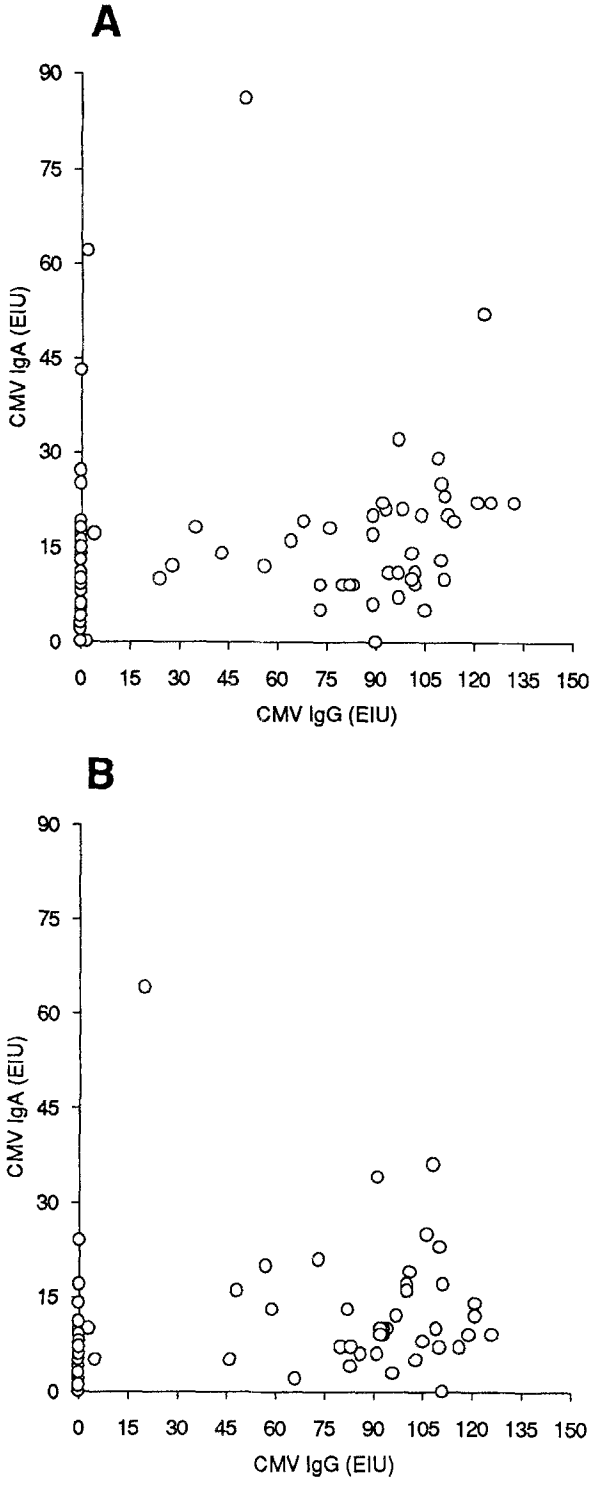

Fig. 2. (**A**, **B**) Plot of CMV IgA antibodies vs CMV IgG antibodies: (**A**) 90 newly-diagnosed IDDM patients; (**B**) 90 age and sex-matched control subjects

tion may have occurred very early in life, or even before birth, and the signs of the infection may already be lost by the time of the diagnosis of diabetes. The onset of clinical diabetes might also promote reactivation of CMV which may result in elevation of antibody levels in newly-diagnosed patients. Therefore, we carried out a prospective time-series on siblings of diabetic children during several years before the manifestation of clinical IDDM. This was possible due to the unique collection of sera during the follow-up of originally healthy siblings of newly-diagnosed IDDM patients.

Cytomegalovirus can be transmitted *in utero* as a consequence of both primary and reactivated maternal infections [16]. Primary CMV infection during pregnancy poses a 30–50 % risk of intrauterine transmission [17, 18], whereas this seems to be very rare in reactivation of latent infections [17]. Fetal infection is more virulent when acquired during the first half of gestation when it may lead to the congenital CMV syndrome [17, 18]. In a Swedish prospective study of congenital CMV infection no evidence was found for congenital CMV infection and IDDM being related [19]. We found no evidence of primary maternal CMV infections during the first 3 months of pregnancy in mothers who gave birth to a subsequently diabetic child suggesting no role of *in utero* CMV exposure in the pathogenesis of IDDM. Further support for this finding was obtained by studying a subgroup of very young diabetic patients diagnosed before the age of 2 years in which we observed no excess of CMV IgG or signs of recent primary CMV infections. In fact, among these very young children the control subjects were more often positive for CMV IgG than the patients.

No signs of primary CMV infections were seen preceding the diagnosis of IDDM or ICA seroconversions during the follow-up of initially healthy siblings of IDDM patients. More than a third of the observed siblings (37 %) had never contracted CMV according to negative CMV IgG and still, they turned ICA seropositive or presented with IDDM. Half of the siblings who contracted diabetes during the follow-up were constantly seronegative as were also 53 % of the newly-diagnosed diabetic children. There was also no correlation between ICA and CMV antibodies. The majority (75 %) of the siblings who became ICA positive during the follow-up had already been infected by CMV before the start of the follow-up, and the rest of them were constantly seronegative. Accordingly, in these siblings primary CMV infections were not temporarily associated with ICA seroconversions. Seroprevalence for CMV IgG was relatively high in pregnant mothers (78 %) and in the very young (31 %) diabetic children. The frequencies of CMV IgG seropositive individuals were similar to those reported earlier in the Finnish population indicating that about 40 % of children have had CMV infection already during their first year of life [20].

Taken together no evidence was found that primary CMV infections could promote or precipitate IDDM. We conclude that if CMV has a role in the pathogenesis of IDDM at all, it is limited to a small proportion of subjects.

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