

Relationship between Human Cytomegalovirus Glycoprotein B Genotype and Serum Alanine Aminotransferase Elevation in Infants

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TERABE, K., SUGIYAMA, K., GOTO, K., MIZUTANI, F., WADA, Y., YOKOYAMA, T. and ANDO, Y. *Relationship between Human Cytomegalovirus Glycoprotein B Genotype and Serum Alanine Aminotransferase Elevation in Infants*. Tohoku J. Exp. Med., 2004, **203** (4), 339-344 — The glycoprotein B (gB) region of the human cytomegalovirus (HCMV) is a major envelope glycoprotein that is a principal target of neutralizing antibodies and is known to stimulate the immune response of cytotoxic T lymphocytes. HCMV is currently classified into four genotypes on the basis of the nucleotide sequence of the gB region. The presence of HCMV in patients under 3 years of age was determined by subjecting urine samples taken from the patients to polymerase chain reaction (PCR) analysis. Analysis by direct sequencing of the gB region was carried out in 90 cases. These cases were grouped into the gB genotype 1 and gB genotype 3. Of 28 cases with a peak serum alanine aminotransferase (ALT) level (≥ 100 IU/l), the duration of observed serum ALT elevation in the gB genotype 1 patients (166.7 ± 126.7 days [mean \pm s.d.] [19 cases]) was significantly longer than that in the gB genotype 3 patients (39.7 ± 31.7 days [9 cases]) ($p < 0.01$). In the 54 cases with a serum ALT level (≥ 50 IU/l), similar tendency was seen ($p < 0.05$). These findings suggest that when serum ALT elevation is confirmed in young children infected with HCMV, analysis of the gB region is helpful for prediction of the duration of serum ALT elevation in the early stage of infection. — human cytomegalovirus; glycoprotein B; alanine aminotransferase; infant

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Many children are infected with HCMV through the placenta, birth canal, breast milk, or horizontal transmission after birth. In infancy or early childhood, HCMV infection is reported to be subclinical, but serum alanine aminotransferase (ALT) elevation is often observed in this period. In some of these cases, protracted serum ALT elevation has been reported (Chiba 1998). When blood examination reveals elevated serum ALT in patients suffering from HCMV infection, it is clinically meaningful to determine whether the elevation has the potential to become protracted. To date, there has been no simple method to predict this in the early stage of infection.

The glycoprotein B (gB) region of the HCMV is a major envelope glycoprotein that is a principal target of neutralizing antibodies (Navarro et al. 1997; Hebart et al. 1997; Meyer-Konig et al. 1998; Peek et al. 1998) and one that is known to stimulate the immune response of cytotoxic T lymphocytes (Hopkins et al. 1996). Although numerous studies have identified the heterogeneity of HCMV, the impact of these heterogenic strains on the pathogenesis of HCMV remains unclear. In the present study, we investigated patients under 3 years of age. We analyzed HCMV DNA taken from the urine samples in order to determine the nucleotide sequence of the gB region and assessed the relationship between the genotype and the degree and duration of observed serum ALT elevation.

MATERIALS AND METHODS

Patient selection

Cases were chosen from among all children under 3 years of age who had visited the Department of Pediatrics at Nagoya City University Hospital and affiliated hospitals between October 1996 and September 1999. Prior to this, the study protocol was approved by ethical review committee at Nagoya City University Medical School. Patients who showed serum ALT elevation and positive HCMV DNA in their urine were selected. But, all patients were screened to eliminate clear cases of marker positive viral

hepatitis, metabolic disease, or malignant tumors. Patients ultimately enrolled as subjects were all those determined to be asymptomatic except biochemical abnormalities. Also, patients who showed serum ALT with a level of below 50 IU/l and positive HCMV DNA in their urine were selected as a control group. All patients were followed without specific therapy, with the exception of cases in which serum ALT values were over 200-300 IU/l and were treated with intravenous injection of Stronger Neo-Minophagen C (glycyrrhizin) until serum ALT decreased to 100-200 IU/l.

The 90 cases chosen for this study included 54 cases in which serum ALT values were over 50 IU/l and 36 cases where serum ALT values were below 50 IU/l. The 54 subjects consisted of 24 males and 30 females with an average age of 0.65 years (range, 0.15-2.96 years). On the other hand, the 36 subjects consisted of 18 males and 18 females with an average of 0.83 years (range, 0.21-3.0 years). No significant differences in any of the tested parameters were observed between the two groups. The post-examination serum ALT was monitored for over 3 months, and when the serum ALT value was below 50 IU/l for more than 1 month in these cases, it was judged to have normal serum ALT value and the duration of observed serum ALT elevation was calculated.

Sample preparation for PCR

Cryopreserved urine (-20°C) samples were thawed immediately prior to PCR. In order to eliminate PCR inhibitory substances from the urine, an Ultrafree MC Filter Unit 100K (Millipore, Bedford, USA) was used to ultrafilter the samples (7000 rpm, 20 minutes). The ultrafiltrated urine was dissolved in 200 µl of distilled water and 2 µl was used for PCR (Khan et al. 1991).

PCR Amplification of gB Gene

Primer synthesized according the published HCMV sequence by Shepp et al. (1996) (First PCR; 5'-CTGGGAAGCCTCGGAACG,

5'-ACCCATGAAACGCGCGGC, Second PCR; 5'-ACGTACTATCCGTTCCGA, 5'-GGCAATCGGTTTGTGTA) was used and a DNA Thermal Cycler (TAKARA, Otsu) was used for nested-PCR. A PCR buffer solution (10 mM Tris HCl, pH 8.3; 1.5 mM MgCl₂; 50 mM KCl) was added to 2 μl of the specimen with 125 μM dNTP, 0.5 unit Taq polymerase, and 0.5 μM primer each to produce a 20 μl reaction system. The reaction time for one cycle was 1 minutes at 94°C (denaturation), 1 minutes at 55°C (annealing), and 2 minutes at 72°C (extension). A final extension was 10 minutes at 72°C. Two μl of the first PCR product was used for the second PCR. First-round PCR was performed for 35 cycles and second-round PCR for 30 cycles.

Detection of amplification products

Electrophoresis of 5 μl of PCR products on a 3% agarose gel was performed. The amplified products were then stained with 0.5 μg/ml ethidium bromide in order to confirm the presence of the target bands under UV light.

Sequencing strategy

In positive cases, the DNA obtained from the 2% agarose gel for electrophoresis was purified using a QIAquick Gel Extraction Kit (QIAGEN, Tokyo). This was followed by sequence reaction according to the manufacture's instructions for BigDye Primer Cycle Sequencing and FS Ready Reaction Kit (Applied Biosystems, Chiba). After the sequence reaction, specimens were analyzed by an ABI PRISMTM 310 Genetic Analyzer (Applied Biosystems, Chiba). The analyzed nucleotide sequences, together with the base sequence of four HCMV strains registered at Gen Bank, were employed as the consensus sequence. An analysis program (ODEN, ver. 2.0) (Ina 1994) was then used to create a molecular dendrogram of the 520 base sequence (gB bp 1077-1596) that included the variable region (condon 440-460) (Chou and Dennison 1991) of the gB domain. The gB type was determined by the phylogenetic analysis.

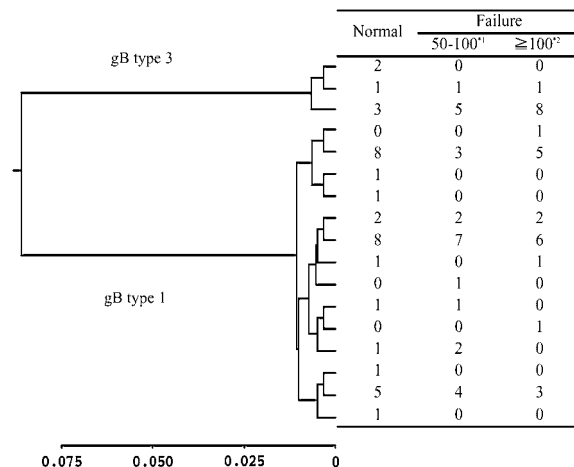
Statistical analysis

The data were analyzed by Fisher's exact probability test or the Mann-Whitney's U-test. A p-value of <0.05 was considered statistically significant.

RESULTS

The 54 cases with a peak serum ALT value ≥50 IU/l were classified into two broad groups based on the molecular dendrogram of the gB region (Chou and Dennison 1991) (Fig.1). A total of 39 cases were confirmed to be classified as gB genotype 1 and 15 cases as gB genotype 3, based on the Chou & Dennison's classification. On the other hand, a peak ALT value <50 IU/l was observed in 30 of 36 (83.3%) gB genotype 1 cases and in 6 of 36 (16.7%) gB genotype 3 cases. However, these differences were not statistically significant between the two groups.

A comparison of peak ALT values among the 54 definite cases of serum ALT elevation revealed that gB genotype 1 was 241.1±318.9 IU/l and gB genotype 3 was 223.2±334.0 IU/l during the observation period, which was not significantly



Phylogenetic analysis of all 90 cases.

Fig. 1. Phylogenetic analysis showing the presence of numerous different genotypes for each gB genotype. Data represent No. of patients. Normal, Normal Liver Function; Failure, Liver Function Failure. *1: Peak ALT value is below 100IU/l. *2: Peak ALT value is above 100IU/l.

different. However, the duration of observed serum ALT elevation was significantly longer in gB genotype 1 patients (102.3 ± 115.4 days) than gB genotype 3 patients (34.3 ± 37.8 days) ($p < 0.05$). The patients were further divided into those with a peak serum ALT values less than 100 IU/l and those greater than 100 IU/l during the period of observation. Among patients with a peak serum ALT less than 100 IU/l (total 26 cases, gB genotype 1, 20 cases; gB type 3, 6 cases), the duration of serum ALT elevation in gB genotype 1 patients was 41.2 ± 57.9 days, whereas it was 26.2 ± 47.6 days in gB genotype 3 patients. Although the duration was shorter among gB genotype 3 patients, both groups showed improved liver function test results after a relatively short period of time and the differences were not statistically significant. In contrast, among patients with a peak serum ALT greater than 100 IU/l (gB genotype 1, 19 cases; gB genotype 3, 9 cases), the peak ALT values for gB genotype 1 patients was 419.2 ± 386.5 IU/l, while gB genotype 3 patients was 327.6 ± 405.6 IU/l. These differences were not statistically significant (Fig. 2). On the other hand, the duration of observed serum ALT elevation in gB genotype 1 patients was 166.7 ± 126.7 days, whereas it was 39.7 ± 31.7 days in gB genotype 3 patients (Fig. 3); a significant difference indicates that the duration is markedly longer in gB genotype 1 patients ($p < 0.01$).

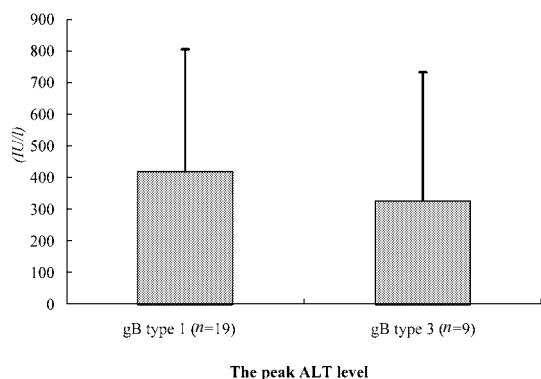


Fig. 2. The peak ALT level among patients with a peak ALT greater than 100 IU/l. These differences were not statistically significant. gB; glycoprotein B. ALT; alanine aminotransferase.

The phylogenetic analysis of all 90 cases indicated the presence of numerous different genotypes for each gB genotype (24 gB genotype 1 species and 8 gB genotype 3 species). Even when the bases were displaced with amino acids, there were still 14 gB genotype 1 and 3 gB genotype 3 subtypes present (Fig. 1). However, no clear relationship could be established among these subtypes and peak serum ALT values and/or the duration of observed serum ALT elevation.

DISCUSSION

Cellular and humoral immunity in HCMV infection reportedly plays a vital role in resistance at the time of primary infection and recovery from infection as well as in determining the severity of a patient's condition. Therefore, the gB region that is one of the targets of neutralizing antibodies (Navarro et al. 1997; Schoppel et al. 1997) and cytotoxic T lymphocytes may be involved in the clinical manifestation of HCMV infection (Hopkins et al. 1996). HCMV is currently classified into four genotypes on the basis of the nucleotide sequence of the gB region (Chou and Dennison 1991). In the present study, phylogenetic analysis of the 520 bases (gB bp 1077-1596) including the gB domain's variable region (codon 440-460), revealed that all of the cases examined belonged to either gB genotype 1 or gB genotype 3.

Previous studies have compared fatal and non-fatal cases of HCMV infection in bone marrow grafts, and have shown a high presence of gB genotype 1 HCMV in non-fatal cases (Fries et al. 1994; Torok-Storb et al. 1997). Furthermore, in patients with advanced human immunodeficiency virus disease, a correlation has been reported between the presence of HCMV gB genotype 2 in the blood and the development of HCMV retinitis (Shepp et al. 1996). Another report on liver transplants noted a high incidence of acute rejection when gB genotype 1 was present (Rosen et al. 1998). On the other hand, another study of organ transplant patients demonstrated an association between T cell affinity to HCMV and gB

genotype (Meyer-Konig et al. 1998). However, they were unable to demonstrate any similar definite relationship between clinical symptoms and gB genotype. Similarly, another study of kidney transplant patients also failed to confirm any definite relationship between clinical symptoms and gB genotype (Vogelberg et al. 1996). At present time, a great deal of controversy remains concerning the relationship between gB genotype and clinical symptoms (Hebart et al. 1997; Peek et al. 1998; Binder et al. 1999).

We found that a significantly longer duration of serum ALT elevation was observed among gB genotype 1 patients than among gB genotype 3 patients ($p < 0.05$). This trend was much more prominent in cases where the peak ALT values exceeded 100 IU/l during the observation period ($p < 0.01$) (Fig. 3). Although fundamental reason of this correlation remains unclear in the present study, the possible mechanism may be due to different gB proteins or linked change in the other HCMV proteins and/or due to host differences in the immune response.

In the present study, HCMV DNA was not obtained directly from liver tissue but from the urine. In the six cases where serum ALT elevation continued over an extended period, the HCMV strain in the urine matched that in the blood and

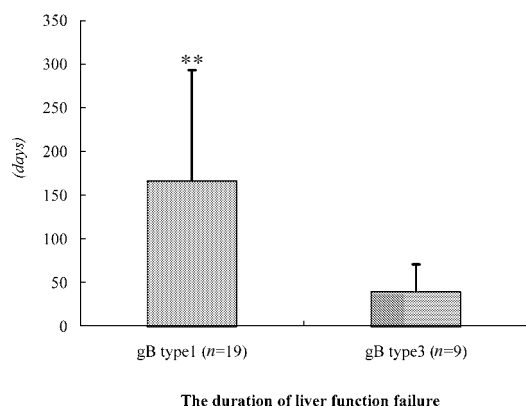


Fig. 3. The duration of liver function failure among patients with a peak ALT greater than 100 IU/l. The duration is markedly longer in gB genotype 1 patients ($p < 0.01$). gB; glycoprotein B. ALT; alanine aminotransferase.

that all were simple clones (data not shown). However, although genotype of HCMV was same between the two different specimens, the examination of liver specimens is necessary to confirm the document HCMV hepatitis. Therefore, further study is necessary to examine liver biopsies to determine whether differences in the gB region may be responsible for the phenotypic differences observed. Furthermore, contrary to above hypothesis, we analyzed genetic polymorphism in a region of the major envelope glycoprotein gB. This region represent less than ~0.2% of the nucleotide sequence of HCMV, so it is only a gross estimate of genetic relatedness. It was possible to conclude that the persistent elevation of ALT was a result of a linked HCMV gene rather than variation in gB gene. Even so, for use in clinical practice, we propose that genetic analysis of gB region of HCMV provides a useful method for predicting the duration of serum ALT elevation in young children with HCMV in the early stage of infection.

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