PATHOGENESIS AND ANTIBODY RESPONSE TO A CYTOMEGALOVIRUS INFECTION IN NEWBORN RATS

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Summary. – The present study described the kinetics of Rat cytomegalovirus (RCMV) infection in newborn rats by monitoring infectious virus and viral antigens in various organs, viral DNA in the blood (DNAemia) and antibody response. These parameters were evaluated quantitatively using double-antibody sandwich ELISA (DAS-ELISA), real-time PCR, indirect ELISA and virus infectivity assay. For the first time DAS-ELISA was used for detection of RCMV antigen directly from organ samples. The relationships between the presence of viral antigens in the infected organs and antibody levels were established by the Spearman's rank test. It was found that the virus was present in the blood, spleen, liver, lungs, and kidneys earlier than in the salivary glands. Furthermore, the early immunity of the newborn rats led to a delayed seroconversion. We suggested that the prolonged presence of the virus in salivary glands could augment the antibody response that conversely might be responsible for a reduction of viremia. This study expanded our understanding of RCMV pathogenesis leading to improved therapeutic and preventive treatment regimens particularly for the neonatal Human cytomegalovirus (HCMV) infections. Additionally, the detection procedures developed in this study such as DAS-ELISA and real-time PCR could serve as alternative techniques for rapid screening of large number of samples.

Key words: DAS-ELISA; indirect ELISA; newborn rats; pathogenesis; Rat cytomegalovirus; real-time PCR

Introduction

Infection with HCMV (the species *Human herpesvirus 5*, the genus *Cytomegalovirus*) can lead to a severe disease manifestation in immunocompromised patients including newborn infants (Alford and Britt, 1993). Congenital or random HCMV infections of premature and full-term infants may result in severe clinical symptoms as reduced respiratory function, hepatosplenomegaly, grey appearance, thrombocytopenia, lymphocytosis and hemolytic anemia (Sever, 2002). HCMV has a narrow host range and cannot be studied in an animal model. Thus, mouse CMV (MCMV, the species *Murid herpesvirus 1*, the genus *Muromegalovirus*) and RCMV (the species *Murid herpesvirus 2*, the genus *Muromegalovirus*), strains Maastricht and English have been widely exploited for decades as surrogate systems to understand pathogenesis, immune response, reactivation and latency of HCMV (Staczek, 1990). Apparently, the pathogenesis of RCMV in newborn rats resembled closely to that of premature infants and young children in humans (Kumar *et al.*, 1984).

Recently, we isolated a new RCMV strain ALL-03 from the rat placenta and uterus (Loh *et al*., 2003). As this strain was unique in its ability to cross placenta and to cause fetal infection (Loh *et al*., 2006), it was an excellent model for

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Abbreviations: CMV = cytomegalovirus; c_t = threshold cycle; DAS-ELISA = double-antibody sandwich ELISA; HCMV = Human cytomegalovirus; i.p. = intraperitoneally; MCMV = Mouse cytomegalovirus; p.i. = post infection; r = Spearman correlation coefficient; REF = rat embryonic fibroblast; RCMV = Rat cytomegalovirus

study of congenital and neonatal infections. Congenitally infected pups were stillborn or died shortly after birth (Bravo *et al.*, 2003). Thus, interactions between RCMV and host as well as prerequisites of animal experiments should be examined very precisely.

The objectives of this study were to describe the time course of acute RCMV infection and to determine the relationships between pathogenesis and antibody response in neonatal rats. The newborn rats were infected with RCMV and the presence of viral DNA, viral antigens and the seroconversion were evaluated at different time intervals post infection (p.i.). For that purpose, the virus infectivity assay, DAS-ELISA, real-time PCR, and indirect ELISA were employed. In addition, since weight loss and splenomegaly were important characteristics of the neonatal HCMV infection in humans (Boppana *et al.*, 1992), the effects of RCMV infection on the spleen and total body weight of animals were also determined.

Materials and Methods

Virus. RCMV strain ALL-03 was employed (Loh *et al.*, 2003).

Animals. Seven-day-old Sprague-Dawley newborn rats tested RCMV-seronegative were used. The pups were nourished by their dams until weaning on day 21 post-partum.

Experimental design. A group of 56 newborn rats was inoculated intraperitoneally (i.p.) with 10^6 $TCID_{50}$ of RCMV. Another group of 52 newborn rats was mock-infected i.p. with PBS. Both groups of rats were checked twice a day for signs of disease. Every 4 days until day 40 p.i., all rats were weighed and four rats from either group were sacrificed. Blood samples were collected for testing of antibodies, viremia and virus infectivity. Various organs (salivary glands, lungs, spleen, liver and kidneys) were taken for examination of the lesions, testing of virus infectivity and use as antigens in DAS-ELISA procedure.

Calculation of spleen to body weight ratio. Spleen weight (g) divided by body weight (g) and multiplied by 1000 gave spleen to body weight ratio.

Indirect ELISA for antibody detection was done as described (Loh *et al*., 2006). Cut-off value was determined by the mean A_{650} + 3 SD of the negative sera. To generate a standard curve, serial 10-fold dilutions of reference serum were plotted against the mean A_{650} + 3 SD. From each A_{650} reading a cut-off value was subtracted. Titers of tested sera were calculated from the regression equation derived from the standard curve in standard manner using net A_{650} values.

DAS-ELISA for antigen detection. Salivary glands (submandibular, parotid and sublingual), lungs, liver, spleen, and kidneys from both infected and mock-infected rats were weighed and homogenized in appropriate volume of PBS to give a final concentration of 100 mg/ml. Organ suspensions were sonicated, clarified and supernatant fluids were used as antigens. Wells of microtiter plates were coated overnight with mouse hyperimmune serum against RCMV diluted 1:100, washed with 0.05% Tween 20 in PBS (PBST) and incubated in blocking buffer (5% BSA in PBST) overnight. Tissue supernatants diluted 1:50 in blocking buffer were added to wells and incubated for 2 hrs. Then, the wells were incubated with rat hyperimmune serum diluted 1:200 in blocking buffer containing 0.5 mol/l NaCl. After incubation for 2 hrs, the plates were incubated with peroxidase-conjugated goat anti-rat IgG diluted 1:2,000 for 2 hrs. This was followed by the addition of tetra methyl benzidine as substrate onto the plates. From each A_{650} reading a cut-off value (a mean A_{650} for extracts from the respective organ of uninfected animals) was subtracted. To generate a standard curve, A_{650} values were plotted against serial two-fold dilutions of a purified RCMV (0.0625–8 μg/ml). The amount of viral antigen in a given sample was calculated from a regression equation derived from the standard curve using mean net A_{650} values in a standard manner. The amount of viral antigen in the given organ was expressed as μg of viral antigen per g of tissue.

Quantitative real-time PCR for DNAemia detection. Buffy coat cells were obtained from the blood and an aliquot of 10^6 cells were subjected to DNA isolation procedure using DNAzol® Genomic DNA Isolation Reagent (Molecular Research Centre). DyNAmo™ SYBR® green qPCR kit (Finnzymes) was used for a quantitative determination of viral DNA load in buffy coat cells. The gene-specific primers used in this assay were flanking the immediate-early 1 gene region of RCMV strain ALL-03 with the sequences stated as follow: 5'-TCACTAACCTGCCACCTATAAC-3' (forward) and 5'-GAAGACATGTAGACGAAGGAGA-3' (reverse). Real-time PCR was carried out by using DNA Engine Opticon™ 2 System. The reaction mixture in volume of 20 ml contained 1x Master Mix, 0.3 mmol/l of each forward and reverse primers, a ten-fold dilution series of RCMV genomic DNA (for standard curve and quantitation) or 5 ml (approximately 250 ng) of each extracted DNA sample and nuclease-free water was prepared on ice. The reaction conditions include an initial denaturation step at 95°C for 10 mins, 30 cycles of 10-secs denaturation at 94°C, 15-secs annealing at 60°C, 15-secs extension at 72°C and fluorescence acquisition step at 75°C for 2 secs. A final extension was done at 72°C for 5 mins. Thereafter, a melting curve analysis was performed. Every real-time PCR assay contained no template controls (NTCs), test samples and a $\log_{10}$ dilution series (0.0025–250 ng) of standard viral DNA for quantitation. The mean threshold cycle (c_t) values of the standard DNA were plotted against the input DNA. The samples represented DNA quantity per 5 x 10^4 buffy coat cells or 250 ng of total genomic DNA. The amount of viral DNA was expressed as pg of viral DNA/μg of total cell DNA.

Virus infectivity assay. A preliminary detection of RCMV in various organs was performed as described (Loh *et al*., 2003). To detect viremia, approximately 10^6 buffy coat cells were co-cultivated with confluent REF cell monolayers and incubated at 37°C for 10 days. When viral plaques were present, cell cultures were regarded as positive. When no plaques were present, cell monolayers were harvested and used in a subsequent passage. Indirect immunoperoxidase staining was used to confirm the infection of REF cells (Loh *et al*., 2003).

Statistical analysis and correlation assessment. The statistical significance of differences between mock-infected and infected groups of data was determined using the two-tailed Student's *t* test. The bivariate correlation matrix amongst antigen levels in tested organs, antibody levels and DNAemia was compiled using non-parametric Spearman's rank test.

Results

Clinical observation and gross pathology

In the control group of animals, no clinical signs were observed indicating illness. In the group of infected animals, clinical signs were not been observed until day 3 p.i. On day 4 p.i. clinical signs became visible and we noticed hemorrhages at the extremities of the limbs and tails, brushy and ruffled hair coat, reduced appetite, loss of the body weight, inactiveness and shivering. Approximately 40% rats became ataxic and lost coordination of their hind legs. However, most of the symptoms disappeared gradually after day 20 p.i. In both groups of rats no mortality was reported throughout the study.

No pathological abnormality was observed in the organs of control animals. In the lungs of 75% of infected rats hemorrhages were detected from day 8 p.i until the end of experiment. Approximately 20% of hemorrhagic lungs were also congested. A few infected rats showed slight congestion and swelling of the liver on days 4, 8, and 12 p.i.

Body weight and spleen to body weight ratio

The average increase of body weight of infected rats was slightly lower when compared to control rats, but the difference was not significant. On the other hand, average spleen to body weight ratio of infected rats increased from 5.1% to 23.2% from day 4 p.i. to day 24 p.i. Thereafter, the ratio increased sharply from 71.8% to 88.0% until day 36 p.i. and finally declined significantly on day 40 p.i. (Table 1). Statistical analysis revealed the significant increase on days 8 ($p < 0.05$), 28 ($p < 0.01$), 36 ($p < 0.05$) and 40 ($p < 0.01$) p.i.

Table 1. Spleen to body weight ratio for RCMV-infected and control newborn rats

Day p.i.	$Ratio_{inf}$	$Ratio_{control}$	$Ratio_{inf}/Ratio_{control}$ x 100 (%)
0	3.24	3.54	91.5
4	4.20	3.99	105.1
8	4.20	3.47	121.1[a]
12	4.95	4.02	123.2
16	4.39	3.77	116.3
20	3.94	3.32	118.5
24	3.13	2.86	109.6
28	4.54	2.64	171.8[a]
32	5.00	2.66	188.0
36	5.02	2.89	173.8[a]
40	3.43	2.87	119.4[a]

$Ratio_{inf}$, $Ratio_{control}$ = spleen to body weight ratio x 1000. [a]Calculated from significant difference.

Indirect ELISA for antibody detection

The control group of rats was seronegative throughout the study. In the group of infected rats specific antibodies were not detected until the day 4 p.i. Thereafter, a slight increase in antibody titers was observed on day 8 p.i. followed by marked increase on day 12 p.i. Antibody titers remained constant from day 16 to day 40 p.i. (Fig. 1). In general, levels of seroconversion against RCMV were low in the infected newborn rats.

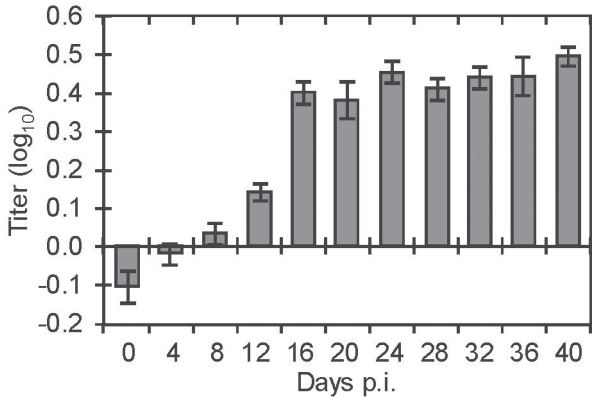

Fig. 1

Antibody titers in sera of infected newborn rats detected by indirect ELISA

Each bar represents geometric mean value of 4 rats ± SD.

DAS-ELISA for antigen detection

In the group of infected rats viral antigen in different amounts was present in all organs tested (Fig. 2). Obtained results showed that liver, spleen and lungs were prime targets of the acute viral infection. Beginning the day 4 p.i. the presence of viral antigen in the liver raised steeply with maximum level detected on day 12 p.i. However, antigen levels dropped significantly 4 days later and maintained this level until day 40 p.i. Amount of RCMV antigen in the spleen increased to maximum on the day 8 p.i. and declined gradually until day 20 p.i. Lungs had similar kinetics of infection as spleen, reaching maximum of viral antigen content on day 16 p.i. Low levels of viral antigens were found in kidneys and salivary glands on day 4 p.i. The increase of antigen level in kidneys was rapid, reaching its peak on day 8 p.i. and stayed at a slightly lower level until day 36 p.i. The salivary glands had lowest level of viral antigen up to the day 12 p.i. Subsequently, a sharp rise in the antigen level was observed on day 32 p.i. Salivary glands had the highest concentration of viral antigen exceeding other organs 11- to 26-fold. In general, all organs tested showed a decline in antigen content on days 36 and 40 p.i.

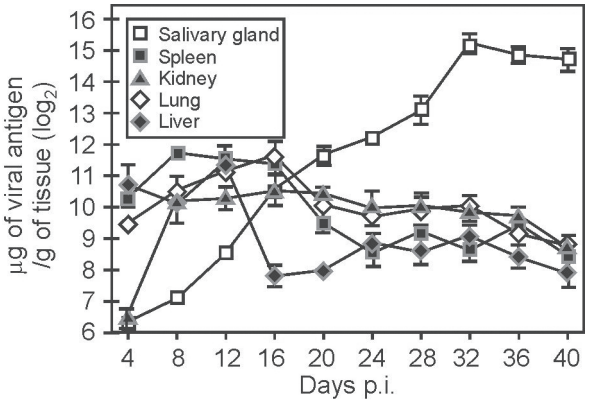

Fig. 2

RCMV antigen distributed in various organs of infected newborn rats determined by DAS-ELISA

Each data point represents geometric mean value of 4 rats ± SD.

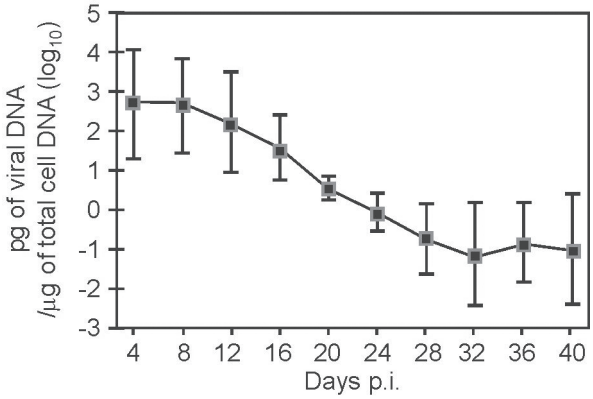

Fig. 3

RCMV DNA load in infected buffy coat cells quantitated by real-time PCR assay.

Each data point represents geometric mean value of 4 rats ± SEM.

Quantitative real-time PCR for DNAemia detection

The amount of viral DNA in buffy coat cells of infected animals was determined on days 4–40 p.i. (Fig. 3). Viral DNA load was highest on day 4 and then declined continuously until day 40 p.i., indicating a more than 200-fold fall (calculated from days 4 and 20 p.i.). After day 24 p.i., some of the samples were negative. Viral DNA load on days 24–40 p.i. was less than 1 pg/µg, which made the significance of DNAemia in RCMV infection dubious.

Detection of infectious virus in various organs and blood

CPE had been observed in each REF cell culture inoculated with infected tissue homogenate, (salivary glands, lungs, spleen, liver, kidneys, buffy coat cells) from day 4 p.i. until day 40 p.i. Obtained results were similar to those published previously (Loh *et al*., 2003). However, mock-infected tissue homogenates did not show any CPE after inoculation of REF cells.

Table 2. Relationships between DNAemia, antibody titer and viral antigen load in different organs evaluated by Spearman's rank test

		DNAemia	Antibodies	Salivary glands	Spleen	Kidneys	Lungs	Liver
DNAemia	R	1.000	–0.764	–0.855	0.727	0.291	0.427	0.373
	P		****0.006**	****0.001**	***0.011**	0.385	0.190	0.259
Antibodies	R	–0.764	1.000	0.882	–0.400	0.027	–0.155	–0.164
	P	****0.006**		****0.000**	0.223	0.937	0.650	0.631
Salivary glands	R	–0.855	0.822	1.000	–0.327	0.036	–0.082	–0.073
	P	****0.001**	****0.000**		0.326	0.915	0.811	0.832
Spleen	R	0.727	–0.400	–0.327	1.000	0.645	0.782	0.536
	P	***0.011**	0.223	0.326		***0.032**	****0.004**	0.089
Kidneys	R	0.291	0.027	0.036	0.645	1.000	0.918	0.100
	P	0.385	0.937	0.915	***0.032**		****0.000**	0.770
Lungs	R	0.427	–0.155	–0.082	0.782	0.918	1.000	0.345
	P	0.190	0.650	0.811	****0.004**	****0.000**		0.298
Liver	R	0.373	–0.164	–0.073	0.536	0.100	0.345	1.000
	P	0.259	0.631	0.832	0.089	0.770	0.298	

R = Spearman's coefficient.

P = two-tailed significance level.

*Correlation was significant at $p < 0.01$, in bold.

**Correlation was significant at $p < 0.05$, in bold.

The data were obtained from 11 samples tested for antibody titers, antigen load and DNAemia at different time intervals p.i.

Correlation matrix of antibody response and virus distribution

The association of antibody response with viral load in various organs (μg/g of tissue) and viral DNA in the blood (pg/μg total extracted DNA) was presented in a correlation matrix (Table 2). Spearman correlation coefficient (r) showing significance at 0.01 and 0.05 levels was compared. The DNAemia was inversely related to viral load in salivary glands ($p < 0.01$) and antibody production ($p < 0.01$) but linked linearly to viral load in spleen ($p < 0.05$). Correlation between the antibody production and viral load in salivary glands was statistically significant ($p < 0.01$) with strong positive association ($r = 0.882$). Viral loads in the spleen, kidneys and lungs had no significant correlation to any variable. Correlation of kidneys and lungs gave the highest level of relationship, i.e. $r = 0.918$ ($p < 0.01$). However, the liver had no significant correlation to any variable.

Discussion

In the current study, we studied pathogenesis of RCMV infection in the newborn rats. Presented findings indicated that RCMV infection caused rapid dissemination of the virus to spleen, liver and lungs with a slight delay to kidneys, followed by salivary glands. These findings strongly resembled observations made in previous RCMV studies (Priscott and Tyrrell, 1982; Bruggeman *et al.*, 1983, 1985; Stals *et al.*, 1990; Kloover *et al.*, 2002a). In these studies, recovery of the virus from spleen, liver, lungs and kidneys was limited to the early period of infection. In salivary glands, the amount of the virus reached its highest point 4 weeks p.i. However, there was no virus detected in kidneys at any time of infection with RCMV strain Maastricht (Kloover *et al.*, 2002a). In contrast, kidneys were a significant target for the infection with our strain ALL-03, as the virus was detected in kidneys as early as on day 4 p.i. In the case of congenital HCMV infection, affected organs were tested in still-borne or deceased children and specific viral antigens were demonstrated in almost all organs (Kasprzak *et al.*, 2000). Conversely, symptoms in life-borne infants were restricted to a few organs, mainly lungs, liver, spleen, bone marrow and central nervous system (Ko *et al.*, 2000). In congenitally infected children large amount of HCMV in urine pointed to a rapid replication of the virus in kidneys (Kasprzak *et al.*, 2000). In general, pathogenesis of the RCMV infection mimicked most of the features established in HCMV infection.

The finding that weight loss in the infected group was not significant when compared to the control group was in agreement with the previous study of RCMV infection (Bruggeman *et al.*, 1985) but contrasted to a guinea pig CMV infection by which the lack of weight gain was an important characteristic (Bravo *et al.*, 2003). Determination of ratio (spleen to total body weight) showed that there was only a slight increase in the ratio at the beginning of infection, which became more pronounced after 24 days p.i. Increase in spleen to body weight ratio coincided with the detection of RCMV in this organ. Similarly, MCMV studies have shown a significant enlargement of the spleen as well as high virus titers detected in this organ (Loh and Hudson, 1981). Nevertheless, splenomegaly is one characteristic of CMV infection.

After the day 16 p.i. antibodies against RCMV were detected, but the antibody titer was extremely low. That fact could be explained by the development of immune response in the newborn rats, as the antibody level increased after day 36 p.i. In previous studies (Bruggeman *et al*., 1983, 1985) specific antibodies were detected in adult rats on day 7 p.i. and their level increased with advanced infection. In the RCMV-infected rats as well as in MCMV-infected mice, IgM antibodies were detected on day 3 p.i., while IgG antibodies were present on days 5–7 p.i. followed by the increase of titers (Lawson *et al.*, 1988). Also in this study, IgG antibodies were detected after day 4 p.i.

DNAemia in significant level was detected on days 4–20 p.i. Later in infection viral DNA level was very low with some rats reaching zero level. Due to high sensitivity of the real-time PCR assay, the completely cleared viremia could not be proved. The circulation of the infected leukocytes in the blood was probably responsible for the dissemination of active virus to the target organs what was in agreement with previous findings (Bruggeman, 1993; Revello *et al.*, 2001). During the viremic phase, CMV DNA is readily detectable in monocytes, lymphocytes and neutrophils (Revello *et al.*, 2001). The systemic spread of HCMV via productively infected circulating blood leukocytes was a hallmark of dissemination of infection, closely linked to development of HCMV disease at an early stage (Saltzman *et al.*, 1992).

The relationship between virus distribution, DNAemia and antibody response showed that the acute infection occurred in blood, spleen, kidneys and lungs and slightly later in salivary glands. The spleen, kidneys and lungs had a linear relationship in viral tropism *in vivo*. However, viral antigens in liver should have a link to spleen, since involvement of these two organs in infection was commonly seen (Priscott and Tyrrell, 1982; Bruggeman *et al.*, 1985). Nevertheless, we found only a moderate correlation between these two organs ($r = 0.536$) that was statistically not significant.

Immunoglobulins present in blood are important tools in controlling blood-borne spread of HCMV and in limiting spread of virus from blood to the fetus in pregnant women who experience HCMV reactivation (Pass *et al.*, 1999). This

was clearly demonstrated from the inverse correlations between leukocyte-borne DNAemia and antibody response in our current study. It was suggested that the ongoing rise in antibody production might help to clear the RCMV-bearing leukocytes from the circulation. In fact, early studies demonstrated that CMV-specific cytotoxic T lymphocytes as well as natural killer cells were also important for recovery from CMV infection and prevention of the recurrence (Polic *et al.*, 1996; Hengel *et al.*, 1998). In concordance with a previous study, we assumed that virus present in salivary glands was responsible for the production of high levels of anti-RCMV IgG antibodies (Kloover *et al.*, 2002b). Newborn mice that have survived MCMV infection established a long-lasting persistence in salivary glands and shed the virus in saliva for several months before termination of productive infection and establishment of latency (Reddehase *et al*., 1994). Therefore, it was predicted that the infection in salivary glands persisted for a definite time and the antibody production increased until a maximal level after day 40 p.i. while the RCMV infections in the spleen, kidneys, lungs and liver vanished as described in the previous studies (Priscott and Tyrrell, 1982; Bruggeman *et al.*, 1985; Stals *et al.*, 1990; Kloover *et al.*, 2002b). The infection of salivary glands and antibody level later in infection decreased gradually to an undetectable level. Reaching this stage, the infection approached the alternate stage of virus latency (Krmpotic *et al.*, 2003).

In this study, quantification of viral antigen and DNA rather than quantification of infectious virus was preferred. This was mainly due to the low possibility of RCMV recovery, because the presence of neutralizing antibody caused rapid decrease of infectious virus (Pass *et al.*, 1999). In addition, CMVs produce much more non-infectious particles and structural proteins than infectious virions (Irmiere and Gibson, 1983). RCMV replication cycle *in vitro* is relatively long and conventional virus infectivity assay such as plaque assay is time-consuming, contamination sensitive and lacks the ability to detect low levels of RCMV. The development of DAS-ELISA for detection of RCMV antigen present in different organs eliminated these disadvantages. Moreover, this is the first use of DAS-ELISA method to detect CMV antigen. The development of real-time PCR method for the detection of CMV DNA had several advantages over the plaque assay including: (i) detection of viral DNA derived from non-replicating virus, (ii) high sensitivity and (iii) precise quantification of CMV DNA copies (Vliegen *et al.*, 2003). Real-time PCR analysis also eliminated post-PCR processing as a potential source of error (Karge *et al.*, 1998; Winer *et al.*, 1999).

In conclusion, newborn rats with premature immunity were susceptible to RCMV infection without induced immunosuppression. RCMV infection involved many target organs and shared some pathological features of HCMV infection. In addition, correlations between the pathogenicity and antibody response provided a better understanding of neonatal HCMV infection and served as a model for HCMV infections of preterm (Yeager *et al.*, 1983) and full-term infants (Kumar *et al.*, 1984). Study of RCMV infection would be useful for the evaluation of new antiviral substances and may provide further insight into the pathogenesis and manifestations of congenital and neonatal HCMV infections. Development of detection procedures as DAS-ELISA and real-time PCR could be useful as alternative techniques for rapid screening and testing of large number of samples, thereby diminishing the cumbersome virus isolation.

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