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Investigation of Varicella-Zoster Virus DNA in Lymphocyte Subpopulations by Quantitative PCR Assay

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***Abstract*: To investigate the nature of viremia during the acute phase of varicella, we studied the viral load in nine otherwise healthy children with varicella. Plasma and peripheral blood mononuclear cells (PBMC) were obtained, then PBMC were divided into CD4+T, CD8+T, and B lymphocytes and monocyte/macrophage fractions. The viral DNA in each component was quantified using a real-time quantitative polymerase chain reaction assay. Varicella-zoster virus (VZV) DNA was detected in plasma, PBMC and all subpopulations. The amount of viral DNA was similar in each PBMC subpopulation, suggesting that each lymphocyte fraction and monocytes carry similar amounts of VZV DNA during viremia.**

***Key words*: Varicella-zoster virus, Lymphocyte subpopulations, Quantitative PCR assay**

Varicella (chickenpox) is characterized by febrile illness with pruritic vesicles, and is the result of primary infection with varicella-zoster virus (VZV). The pathogenesis is as follows: virus entry at the respiratory mucosa and primary viremia are believed to occur; then secondary viremia of greater magnitude occurs; finally, the rash results. Understanding the nature of the viremia is thought to be important in order to clarify the pathophysiology of varicella infections and complications of the disease (1, 7). Peripheral blood mononuclear cell (PBMC)-associated viremia has been demonstrated just before and after the onset of disease (2, 5, 6, 13, 15, 16). Determining the phenotypes of the PBMC subpopulations is complicated by the low frequency of positive cells and the difficulty in achieving highly purified preparations of subpopulations.

Recently, we established a quantitative real-time polymerase chain reaction (PCR) assay to quantify the VZV genome copies (8). Using this assay, we observed that there was a higher occurrence of viremic VZV in varicella than in zoster, and that acyclovir treatment markedly suppressed viremia in varicella (9). In this study, we quantified the viral load in the peripheral blood leukocyte subpopulations to determine which subpopulations harbor VZV during the acute phase of varicella. We also quantified the viral load in plasma to investigate whether viral DNA in blood is restricted to PBMC fraction.

Nine healthy children with clinically diagnosed varicella (0 to 3 years old, median: 1 year) were enrolled in this study. None of these patients had a previous history of varicella. At the time of entry, none of the patients had received oral acyclovir therapy.

All the specimens were obtained after informed consent. Blood samples were taken when the patients had clinical indications in the acute phase. Sampling occurred 2 to 5 days after the onset (mean: 2.3 days). Whole blood was obtained from the patients, and plasma and PBMC were separated by density gradient with Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). Then PBMC subpopulations (CD4+, CD8+, CD14+, CD19+) were isolated by positive selection using immunomagnetic beads (M-450 CD4, M-450 CD8, M-450 CD14, and M-450 CD19; Dynal, Oslo, Sweden) according to the supplier's instructions. To confirm the

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Abbreviations: PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; VZV, varicella-zoster virus.

Table 1. Quantitation of varicella-zoster virus DNA in peripheral blood mononuclear cells subpopulations

	Total no.	No. with detect		Log VZV genome numbers (mean ± SD[a])/100 μl or /10^5 cells)	
		gB	ORF62	gB	ORF62
Plasma	5	4	5	3.0±0.5	3.0±0.9
PBMC	9	8	9	1.1±0.7	0.8±0.8
CD4+	9	1	3	1.4±0.0	1.5±0.4
CD8+	9	4	3	2.1±0.7	2.1±0.3
CD14+	6	2	3	2.3±0.4	1.6±1.0
CD19+	8	5	3	1.9±0.6	0.6±1.4

[a] SD, standard deviation.

purity of each PBMC subpopulation, immunomagnetic beads were detached from isolated cells by using Detachabeads for M-450 CD4, M-450 CD8, and M-450 CD19 (Dynal). Aliquots of 1×10^5 isolated cells were incubated with Opticlone CD4-fluorescein isothiocyanate/CD8-phycoerythrin or CD3-fluorescein isothiocyanate/CD19-phycoerythrin (Immunotech, Marseilles, France). Cell samples were analyzed on FACScan (Becton Dickinson, Mountain View, U.S.A.) using CellQuest software (Becton Dickinson). The purity of each PBMC subpopulation was confirmed by flowcytometry analysis. All the purity results were greater than 92%.

The real-time quantitative PCR assay was performed using a TaqMan PCR kit and a Model 7700 Sequence Detector (PE Applied Biosystems, Foster City, U.S.A.), as previously described (8, 9). Primers to amplify the glycoprotein B (gB) and ORF62 genes were selected. Briefly, DNA solution was added to a PCR mixture containing each primer, fluorogenic probe, and AmpliTaq Gold (PE Applied Biosystems). Following activation of the AmpliTaq Gold for 10 min at 95 C, 50 cycles of 15 sec at 95 C and 1 min at 60 C were carried out on the Model 7700 Sequence Detector. Real-time fluorescent measurements were taken and a threshold cycle value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit. Standard curves were constructed using the values obtained from serially diluted positive control plasmids that contained the gB or ORF62 gene. The values from clinical samples were plotted on the standard curve, and the copy number was calculated by interpolation. Samples were defined as negative when the threshold cycle value exceeded 50 cycles. To normalize the amount of VZV-DNA in PBMC, human β actin DNA was quantified by real-time PCR using TaqMan β actin Control Reagents (PE Applied Biosystems). From the amount of human β actin DNA, the number of PBMC in the DNA solution was estimated, and the number of gB or ORF62 genomes was adjusted and expressed per 10^5 cells. Since ORF62 is diploid in the VZV genome, the copy number was divided by two to obtain the VZV genome copies in samples.

We quantified the viral load in plasma, PBMC and each selected subpopulation by the real-time PCR assay. Either the gB or ORF62 gene was detected in all plasma and PBMC (Table 1). Each PBMC subpopulation was positive for VZV DNA in at least one patient, although the positive rates were variable. CD8+ and CD14+ cells had somewhat larger genome numbers, although the differences were not statistically significant (Mann-Whitney *U* test). The mean VZV genome numbers calculated based on gB and ORF62 genes were roughly comparable in the two groups (Table 1).

The viremia in varicella is thought to be cell-associated (1). However, de Jong et al (4) reported that VZV DNA was detected in serum and plasma from 88.9% patients, and the mean was $10^{3.3}$ copies/ml. Our data gave similar results to their observation. Although further studies are needed to determine the precise source of the viral DNA, it seems that cell-free VZV DNA exists in plasma and serum from varicella patients.

The PBMC-associated viremia in varicella is presumed to allow dissemination of the virus to cutaneous epithelial cells and to result in pruritic vesicles. Therefore, VZV interactions with blood cell types are important for viral pathogenesis. Currently, both lymphocytes and monocyte/macrophages are reported to harbor infectious virus, as determined by isolation of the virus (3, 14) and *in situ* hybridization (10–12). Asano et al (3) reported that VZV was isolated from 71% of non-adherent cells, 43% of T lymphocytes, and 33% of B lymphocytes, but only 5% of monocytes. Whereas, Mainka et al (11) showed that T and B lymphocytes as well as monocytes expressed viral protein (glycoprotein E) on their surface; about two-thirds of the infected PBMC were T lymphocytes, and the remaining third consisted of both B lymphocytes and monocytes. We tried to detect and quantify VZV DNA in each subpopulation using magnetic bead sorting and real-time PCR. We detected viral DNA in CD4+T, CD8+T, and B lymphocytes

and monocyte/macrophage subpopulations. The amount of viral DNA was similar in each subpopulation.

The positive rates of VZV DNA in subpopulations were variable, although most PBMC were positive. In some samples, only a limited number of cells was available for quantitative PCR because of cell loss during the procedure or because of the low percentage of each subpopulation in peripheral blood. Therefore, some of our results might be false negatives. To overcome this disadvantage, we quantified both VZV DNA and human β actin DNA, and corrected and adjusted the number of VZV genomes per 10^5 cells. The amount of viral DNA was similar in each PBMC subpopulation, suggesting that each lymphocyte fraction or monocyte harbors similar amounts of VZV DNA during the acute viremic phase of varicella.

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