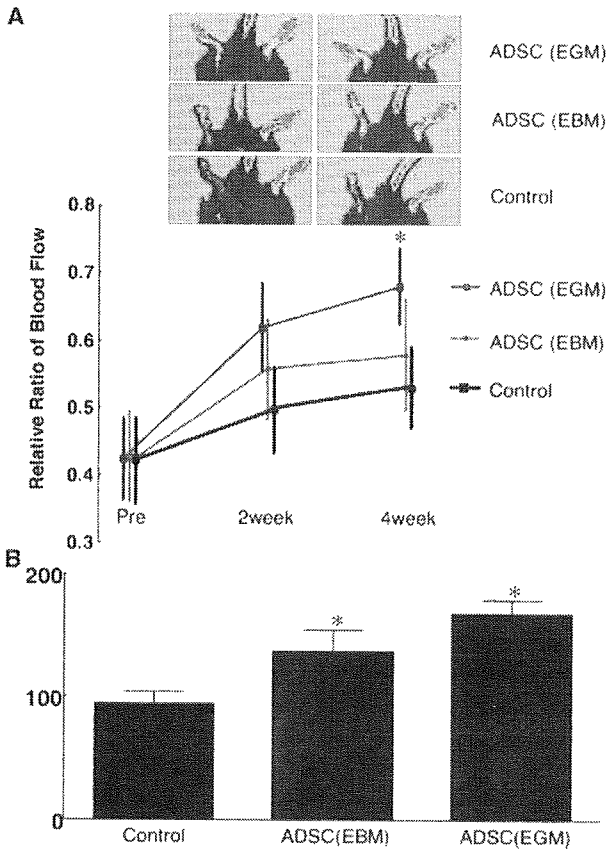

Figure 4. Effect of ADSC injection in mouse ischemic limb model. A, Representative image of peripheral blood flow analyzed by LDI at 2 or 4 weeks after injection. Color-coded images represent blood flow distribution; low or no perfusion is displayed as blue, whereas highest perfusion is displayed as red. Quantitative analysis of blood flow in hindlimb is expressed as perfusion ratio of ischemic hindlimb to untreated opposite limb. B, Capillary density in cross sections of ischemic tissue immunostained with anti-CD31 (PECAM) antibody. Control indicates phosphate-buffered saline injection; ADSC (EBM), indicates ADSC maintained in EBM-2 medium (without growth factor); ADSC (EGM), ADSC maintained in EGM2-MV medium (with growth factor). Each group contains 7 or 8 animals. *$P<0.01$ vs Control.

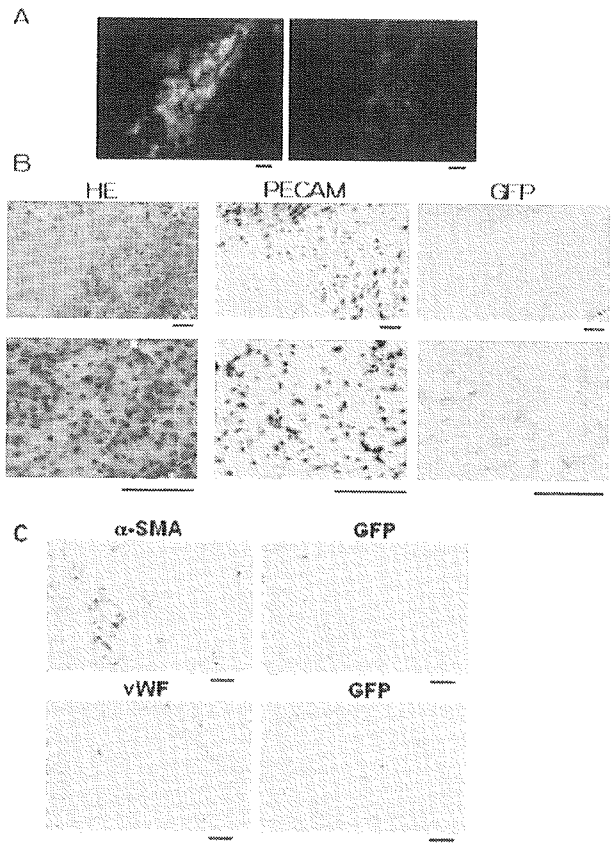

Figure 5. A, GFP-expressing ADSC in cross section of ischemic tissue visualized under a microscope. Left panel indicates 3 days after GFP-expressing ADSC injection, and right panel indicates 14 days after GFP-expressing ADSC injection. Bar=100 μm. B, HE staining (left) and immunostaining with anti-PECAM (center) and anti-GFP (right) antibody in cross sections of ischemic tissue at 28 days after GFP-expressing ADSC injection. Upper panel shows low magnification and lower panel shows high magnification. Bar=100 μm. C, The immunostaining with anti-vWF(von Willebrand factor) or α-SMA(smooth muscle actin) (left) and anti-GFP (right) antibody in cross sections of ischemic tissue at 28 days after GFP-expressing ADSC injection. Bar=100 μm

limitation of primary endothelial progenitor cell transplantation. It was reported that ex vivo expansion of endothelial progenitor cells cultured from the peripheral blood of healthy human volunteers yielded $\approx 5.0\times10^6$ cells per 100 mL of blood, whereas heterologous transplantation requires 0.5 to 2.0×10^4 human endothelial progenitor cells per gram of body weight (of the recipient mouse) to achieve satisfactory reperfusion of the ischemic hindlimb.[27] Actually, the amount of autologous BM blood aspirated for therapeutic neovascularization was approximately 500 mL per person (ie, 0.1% of body weight).[7] This suggests a practical limitation of endothelial progenitor cell transplantation; namely, the volume of blood required to extract an adequate number of endothelial progenitor cells for autologous transplantation. The main benefit of our ADSC is that they can be easily harvested from patients by a simple, minimally invasive method and also easily cultured (1×10^5 cells from one mouse and 20 mL lipoaspiration from a human). Moreover, cultured ADSC can be expanded more rapidly (>10 times within 1 week), and long-term cultured cells after some passages still retain their mesenchymal pluripotency, with expression of *Scal* and CD44. This suggests that ADSC could be a good candidate as a novel source of cell therapy in cardiovascular disease.

Recently, therapeutic endothelial stem cell transplantation was suggested to be a promising approach to restore tissue vascularization after ischemic events.[6] However, the true in vivo differentiation capacity of adult BM stem and progenitor cells and their possible contribution to nonhematopoietic cells and tissues including endothelial cells remain controversial.[28,29] We speculate that the injection of cells are not mimicking strictly the natural course of the cells infused or the cultured stem cells under special conditions can change the properties of these cells in terms of their capacity to incorporate into target tissue. In addition, we have to observe carefully whether the survive cells would be a "differentiation" or "fusion." A very recent report suggested that adult BM-derived cells participate in angiogenesis, and a small subpopulation of them differentiate into vascular mural peri-

endothelial cells that are morphologically indistinguishable from pericytes.[30] BM contains nonhematopoietic stromal cells, which comprise immature mesenchymal stem cells, EPC, fibroblasts, osteoblasts, EC, and adipocytes,[8] and infiltrating inflammatory leukocytes release angiogenic cytokines including VEGF. Interestingly, treatment with anti-VEGF monoclonal antibody completely abolished BM-induced neovascularization.[31] This suggests that stem cell transplantation is also "cell-based cytokine therapy." Of importance, in this study we used media containing growth factors to induce the direction of differentiation into vascular cells, to avoid differentiation into unexpected cells, and also activate the secretion of angiogenic growth factors leading to "cell-based cytokine therapy." Although in this study our ADSC-mediated cell therapy did not show further angiogenic effect in the comparison with BM cell or HGF gene therapy, it would be a study limitation to conclude their effectiveness in this ischemic hindlimb model. Our cultured ADSC can provide stem cells by FACS analysis and are easily available for autologous cell therapy, which would allow feasible implementation of "stem cell-based functional gene therapy" in the future.

Increasing numbers of reports highlight the possibility that adipogenesis and neovascularization are reciprocally regulated and tightly linked in rodents. Our results indicate that ADSC represent an easily accessible cell source with potential effects that can be used for therapeutic angiogenesis through growth factor secretion, so-called adipocytokine.

Acknowledgments

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RESEARCH ARTICLE

Biocompatible polymer enhances the *in vitro* and *in vivo* transfection efficiency of HVJ envelope vector

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Abstract

Background Vector development is critical for the advancement of human gene therapy. However, the use of viral vectors raises many safety concerns and most non-viral methods are less efficient for gene transfer. One of the breakthroughs in vector technology is the combination of the vector with various polymers.

Methods HVJ (hemagglutinating virus of Japan) envelope vector (HVJ-E) has been developed as a versatile gene transfer vector. In this study, we combined HVJ-E with cationized gelatin to make it a more powerful tool and assessed its transfection efficiency *in vitro* and *in vivo*. In addition, we investigated the mechanism of the gene transfer by means of the inhibition of fusion or endocytosis.

Results The combination of both protamine sulfate and cationized gelatin with HVJ-E, referred to as PS-CG-HVJ-E, further enhanced the *in vitro* transfection efficiency. In CT26 cells, the luciferase gene expression of PS-CG-HVJ-E was approximately 10 times higher than that of the combination of protamine sulfate with HVJ-E or the combination of cationized gelatin with HVJ-E, referred to as PS-HVJ-E or CG-HVJ-E, respectively. Furthermore, the luciferase gene expression in liver mediated by intravenous administration of CG-HVJ-E was much higher than the luciferase gene expression mediated by PS-HVJ-E or PS-CG-HVJ-E and approximately 100 times higher than that mediated by HVJ-E alone.

Conclusions Cationized gelatin-conjugated HVJ-E enhanced gene transfection efficiency both *in vitro* and *in vivo*. These results suggest that low molecular weight cationized gelatin may be appropriate for complex formation with various envelope viruses, such as retrovirus, herpes virus and HIV.

Keywords non-viral vector; gene transfer; polymer; fusion-mediated delivery

Introduction

The success of gene therapy is largely dependent on the development of a vector. So far, numerous viral and non-viral (synthetic) methods of gene transfer have been developed and improved upon. The use of viral vectors raises many safety concerns because of the possible co-introduction of genetic elements from parent viruses, leaky expression of viral genes, immunogenicity and changes in the host genome structure [1,2]. Non-viral vectors are less toxic and less immunogenic alternatives to viral vectors [3,4]. However, most non-viral methods are less efficient for gene transfer, especially *in vivo*. Thus,

a breakthrough in vector technology is required for the development of highly efficient vectors with low toxicity.

One promising development in vector technology is the combination of the vector with various polymers [5,6]. Biocompatible polymers have been combined with viral and non-viral vectors to enhance gene transfer efficiency both *in vitro* and *in vivo* [7–12]. Adenovirus vector combined with atelocollagen increased stability in tissues and reduced the toxicity [13,14]. The mixture of adeno-associated vector with heparin increased transfection efficiency [15]. The most popular polymers to enhance transfection efficiency are cationic polymers, such as polyethylenimine [16–19] and cationized gelatin [20–22]. Cationic polymers assemble with vectors and form small composite particles that interact with the cell surface and are internalized by endocytosis. The polymer must be positively charged to increase the transfection efficiency of the polymer–DNA complex (polyplex) [23]. However, cationic polymer-based gene delivery systems have faced limitations in the systemic delivery of therapeutic genes due to difficulties in formation, *in vivo* stabilization, toxicity and low transfection efficiency [24–28]. Moreover, positively charged polyplexes aggregate more readily as their concentration increases, and they quickly precipitate out of solution above their critical flocculation concentration or in the presence of salt or serum. These drawbacks have limited the progress of polyplexes in clinical trials. Recent efforts to solve the limitations of polymers have focused on the development of low molecular weight polymers, biodegradable polymers and polymers with reduced positive charge [29]. Gelatin is a biodegradable polymer with various sizes ranging from high (MW 100 000 Da) to low molecular weight (MW 3000 Da) [30]. By conjugation with cationic molecules (Figure 1), such as ethylenediamine, spermine or spermidine, the positive charge ratio per gelatin molecule can be controlled [20,22].

In the present study, we combined HVJ (hemagglutinating virus of Japan) with cationized gelatin. HVJ envelope vector (HVJ-E) is a unique non-viral vector which incorporates plasmid DNA into inactivated HVJ particles. HVJ, also known as Sendai virus, can fuse with cell membranes [31]. Two distinct glycoproteins on the viral envelope are required for cell fusion. The HVJ RNA genome is approximately 15 kb. When the viral genome is intact, highly immunogenic viral proteins are produced in the infected cells. Therefore, we inactivated HVJ with UV irradiation and incorporated plasmid DNA into inactivated viral particles by mild detergent treatment and centrifugation. The resulting HVJ-E can fuse with cell membranes to directly introduce plasmid DNA into cells both *in vitro* and *in vivo* [32]. The major limitation of HVJ-E is the instability of viral particles in fresh blood. Although this characteristic of HVJ-E is an advantage in terms of safety, it is an obvious defect in terms of efficacy.

In this manuscript, we report that cationized gelatin-conjugated HVJ-E enhances gene transfection efficiency both *in vitro* and *in vivo*.

Materials and methods

Reagents, cells and preparation of DNA

Triton-X 100 was purchased from Nakalai Tesque (Kyoto, Japan) and used as a detergent diluted with TE solution (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) to 3% concentration when we incorporated plasmid DNA into HVJ-E. Gelatin was prepared through an acid process of pig skin type I collagen and was kindly supplied by Nitta Gelatin Co. (Osaka, Japan). Ethylenediamine (ED), glutaraldehyde, 2,4,6-trinitrobenzenesulfonic acid, β-alanine and the protein assay kit (lot no. L8900) were purchased from Nakalai Tesque (Kyoto, Japan) and used according to the manufacturer's instructions. As a coupling agent, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride salt (EDC) was obtained from Dojindo Laboratories (Kumamoto, Japan).

Primary human aortic endothelial cells (HAEC) were purchased from Sanko-Junyaku (Tokyo, Japan). All other cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). Adherent and primary cells were cultured in Dulbecco's modified Eagle's medium

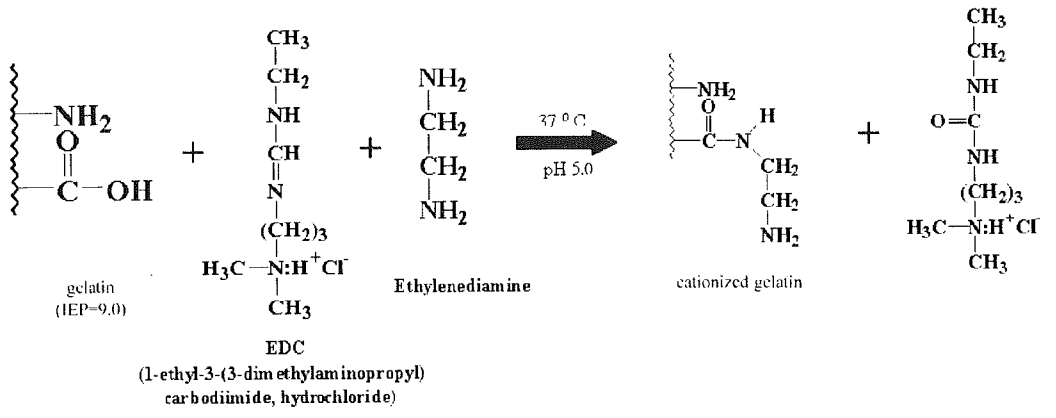

Figure 1. Synthesis of cationized gelatin. Cationized gelatin was mixed with HVJ-E containing a marker gene. The complex was isolated by centrifugation and used for transfection experiments. (IEP; isoelectric point)

(DMEM) and RPMI 1640, respectively, supplemented with 10% fetal bovine serum (FBS).

Luciferase expression plasmid driven by the cytomegalovirus promoter was purchased from Promega (Madison, WI, USA). Qiagen columns (Hilden, Germany) were used to purify DNA.

Preparation of cationized gelatin combined with HVJ-E

HVJ was prepared as previously described [31]. HVJ was propagated in chick eggs, purified by centrifugation, inactivated by UV irradiation and stored at −20 °C as previously described [32]. Stored virus was suspended in 40 μl of TE solution (10 mM Tris-Cl, pH 8.0, 1 mM EDTA). The virus suspension was mixed with plasmid DNA (200 μg/50 μl) and 5 μl of 3% Triton X-100. The mixture was centrifuged at 18 500 *g* for 15 min at 4 °C. After washing the pellet with 1 ml of balanced salt solution (10 mM Tris-Cl, pH 7.5, 137 mM NaCl and 5.4 mM KCl) to remove the detergent and unincorporated DNA, the envelope vector was suspended in 300 μl of phosphate-buffered saline (PBS). The vector was stored at 4 °C until use.

Cationization of gelatin was performed by introducing ethylenediamine (ED) into the carboxyl groups of low molecular weight gelatin (MW 5000) (Figure 1). Briefly, 13.98 g of ED and 2.67 g of EDC were added to 250 ml of 0.1 M phosphate buffer (pH 5.0) containing 5.00 g of low molecular weight gelatin. The reaction mixture was agitated at pH 5.0 at 37 °C for various time periods and then dialyzed against double-distilled water for 48 h at 25 °C by use of a dialysis membrane tube (lot no. 131 096, cut-off MW 1000, Spectra/PorCE, SPECTRUM) to separate residual ED- and EDC-degraded product from cationized gelatin prepared. The dialyzed solution was freeze-dried to obtain powdered cationized gelatin. The percentage of amino groups introduced into this gelatin, referred to as cationized gelatin, was determined by the trinitrobenzenesulfonate method based on the calibration curve prepared by using β-alanine [22]. The percentage of amino groups introduced into gelatin was 48.7 mole/mole carboxyl groups of gelatin.

A complex was formed between the HVJ-E vector and cationized gelatin by simply mixing the two materials in aqueous solution. Briefly, 5 mg of cationized gelatin were added to 300 μl of 0.1 M PBS (pH 7.4) containing 3×10^{10} particles of HVJ-E vector. The solution was mixed by tapping several times. Then, the solution was incubated on ice for 30 min to form cationized gelatin-conjugated HVJ-E vector. The optimal ratio of cationized gelatin and HVJ-E was determined by the measurement of luciferase activity *in vitro*. Cationized gelatin-conjugated HVJ-E vector was purified by centrifugation.

Measurement of zeta potential and apparent molecular size

The zeta potential was measured by an electrophoretic light scattering (ELS) assay. This assay was performed with an ELS-7000AS instrument (Otsuka Electric Co. Ltd., Osaka, Japan) at 37 °C with an electric field strength of 100 V/cm [20]. The ELS measurement was performed 3 to 5 times for each sample. The particle size of HVJ-E or polymer-conjugated HVJ-E was measured by dynamic light scattering (DLS) assay, as previously described [20]. The DLS measurement was performed 3 to 5 times for each sample.

Gene transfer *in vitro* and *in vivo*

For *in vitro* transfection, approximately 5×10^5 cells were prepared 1 day before transfection. HVJ-E ($3–6 \times 10^9$ particles) or cationized gelatin-conjugated HVJ-E was mixed with various concentrations of protamine sulfate. This mixture was added to cells cultured in medium supplemented with 10% FBS. After incubation for 10 min at 37 °C and 5% CO_2, the medium was replaced. The cells were cultured overnight before the gene expression was assayed. For *in vitro* transfection with anionic liposomes, the procedure was as previously described [33]. Luciferase activity was measured with a luciferase assay kit (Promega), and the protein content of the samples was assayed by the Bradford method as previously described [32].

HVJ-E (6×10^9 particles) or cationized gelatin-conjugated HVJ-E containing the luciferase gene (6 μg) was suspended in 100 μl PBS with or without protamine sulfate (200 μg) and injected into the tail veins of BALB/c mice (8 weeks of age). Mice were euthanized 24 h after the injection. The organs including lung, liver, spleen, heart and kidney were removed and cut into small pieces in 5-times volume of diluted luciferase cell culture lysis reagent (Promega). All steps were performed on ice. After centrifugation at 2380 *g* at 4 °C for 10 min, 20 μl of the supernatant were assayed for luciferase activity. All animals were handled in a humane manner in accordance with the guidelines of the Animal Committee of Osaka University.

Assessment of the effect of fusion and endocytosis on transfection efficiency

We prepared antiserum against F protein of HVJ by immunizing a rabbit with purified F protein. The concentration of anti-F antibodies in the antiserum was approximately 30 μg/ml. The aliquots of antiserum were stored at −80 °C. The antiserum was diluted with saline. Polymer-combined HVJ-E (3×10^9 particles) that contained the luciferase gene was preincubated with diluted or undiluted antiserum (20 μl) for 30 min at 37 °C. Then, this mixture was added to cultured

cells. Preimmune rabbit serum was used as a control. Luciferase activity was measured 24 h after the transfection.

Wortmannin (Sigma Chemical Co.) was dissolved in dimethyl sulfoxide to a final concentration of 10 mM, dispensed into 5-μl aliquots and stored at −80 °C. Prior to use, wortmannin aliquots were thawed and diluted in serum-free DMEM. Care was taken to shield the aliquots from light. Before transfection, cells were washed with serum-free DMEM and incubated with various concentrations of wortmannin for 15 min [34,35]. The cells were then subjected to *in vitro* transfection, as described above.

Assessment of the effect of fresh mouse serum on gene transfection with HVJ-E and polymer-conjugated HVJ-E

HVJ-E, PS-HVJ-E, CG-HVJ-E and PS-CG-HVJ-E containing luciferase expression plasmid were separately suspended in 100 μl PBS. The suspensions were mixed with 100 μl of fresh mouse serum. The mixture was incubated at 37 °C for 5 min. Then, after the serum had been removed by centrifugation, the vector, suspended in 30 μl of PBS, was added to cultured cells, and the cells were incubated at 37 °C for 10 min in a 5% CO_2 incubator. The medium was replaced with fresh medium containing 10% FBS. The luciferase activities of each sample were measured 24 h after transfection.

Statistical analysis

The Bonferroni/Dunn test was used to determine whether differences were statistically significant. A value of $P < 0.05$ was considered significant.

Results

Measurement of zeta potential and apparent molecular size

First, we examined the zeta potential and particle size of these complexes (Table 1). HVJ-E was anionic (−3.87 mV), and the diameter was approximately 350 nm. With protamine sulfate, the zeta potential became cationic (4.51 mV), and the diameter was six times larger (2114 nm). The cationized gelatin complex was more cationic (11.30 mV) and smaller (777 nm) than PS-HVJ-E. The zeta potential and size of PS-CG-HVJ-E were intermediate (9.53 mV, 1927 nm) between those of PS-HVJ-E and CG-HVJ-E.

Table 1. Apparent molecular size and Zeta potential of HVJ-envelope vector and its complexes

Complex	Apparent molecular size (nm)	Zeta potential (mV)
HVJ-E	355 ± 35	−3.87 ± 0.69
PS-HVJ-E	2114 ± 207	4.51 ± 0.86
CG-HVJ-E	777 ± 140	11.30 ± 2.52
PS-CG-HVJ-E	1927 ± 292	9.53 ± 1.47

Evaluation of the *in vitro* transfection efficiency of HVJ-E conjugated to cationized gelatin, protamine sulfate or both

Then, we examined the *in vitro* transfection efficiency of HVJ-E, CG-HVJ-E, PS-HVJ-E and PS-CG-HVJ-E. Low molecular weight cationized gelatin (MW 5000 Da) increased the HVJ-E transfection efficiency, but high molecular weight cationized gelatin (MW 100 000 Da) was not effective for gene transfer with HVJ-E (data not shown). As shown in Figure 2, cationized gelatin increased transfection efficiency to the same level as protamine sulfate when compared with HVJ-E alone. An amount of 500 μg of cationized gelatin added to 3×10^9 HVJ-E particles resulted in the highest gene transfection efficiency of CG-HVJ-E without affecting cytotoxicity. When protamine sulfate was added to CG-HVJ-E, the resulting luciferase gene expression in CT26 cells was approximately 10 times higher than the luciferase gene expression mediated by PS-HVJ-E or CG-HVJ-E (Figure 2). The enhanced transfection efficiency resulting from CG-HVJ-E combined with protamine sulfate was also observed in other cell lines (B16-F1) and primary cells (HAEC, human aortic endothelial cells), although the enhancement ratio varied among the different types of cells (Table 2).

Assessment of the effect of fusion and endocytosis on transfection efficiency

Next, the mechanism of transfection by PS-CG-HVJ-E was investigated. To test the effect of fusion protein of HVJ-E on transfection efficiency, the complex was incubated with anti-F protein antibody, and then the mixture was added to cells. As shown in Figure 3A, HVJ-E or CG-HVJ-E was preincubated with anti-F protein antiserum, and the mixture of the vector and serum was added to cultured cells. Luciferase gene expression was hardly detected. Preimmune serum did not cause inhibition. When diluted anti-F serum was used, the luciferase gene expression recovered in a dilution-dependent manner. Dot-blot analysis revealed that 1 μg anti-F antibody bound to 9.7×10^6 HVJ-E particles. From this data, the undiluted antiserum (20 μl) could bind to 5.8×10^9 PS-CG-HVJ-E particles. Therefore, it was anticipated that the undiluted antiserum contained an excess amount of anti-F antibody recognizing all the PS-CG-HVJ-E

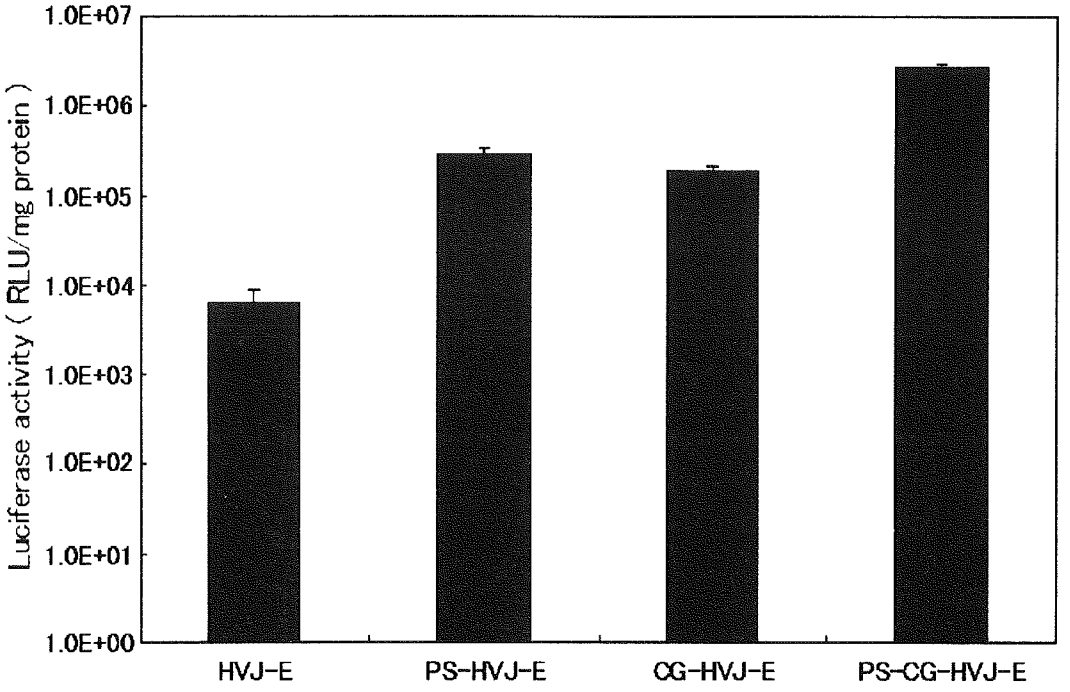

Figure 2. Luciferase gene expression in CT26 cells transfected with HVJ-E, PS-HVJ-E, CG-HVJ-E or PS-CG-HVJ-E. The vectors were incubated with cells for 10 min, and the luciferase activity was measured 24 h after removal of the vector. Results are shown as mean ± s.d. (n = 3). Similar results were obtained in three experiments

Table 2. Results of *in vitro* transfer with Cationized Gelatin conjugated HVJ-envelope vector

Cell line	HVJ-E	PS-HVJ-E	CG-HVJ-E	PS-CG-HVJ-E
Adherent cells				
B16-F1	$7.36 \pm 0.09 \times 10^5$	$8.15 \pm 0.40 \times 10^6$	$7.56 \pm 1.92 \times 10^6$	$1.16 \pm 0.04 \times 10^7$
BHK21	$3.49 \pm 0.38 \times 10^6$	$1.43 \pm 0.05 \times 10^7$	$3.71 \pm 0.18 \times 10^7$	$3.20 \pm 0.30 \times 10^7$
Primary cell				
HAEC	$8.94 \pm 0.88 \times 10^4$	$7.62 \pm 0.55 \times 10^4$	$1.54 \pm 0.06 \times 10^5$	$2.47 \pm 0.82 \times 10^5$

Luciferase activity (RLU/mg protein)

particles used in the experiment, but the antiserum diluted more than 2-fold failed to recognize all the particles. This result was consistent with the data shown in Figure 3A.

Then, the possibility of endocytotic uptake of the complex was assessed using wortmannin, which inhibits endocytosis [34,35]. Wortmannin inhibited the luciferase gene expression in a dose-dependent manner (Figure 3B). Wortmannin at a concentration of 100 nM inhibited gene transfection efficiency by 40%. The inhibition with wortmannin was much smaller than that with anti-F antibody. At the same time, although we tested the affecting cytotoxicity of wortmannin, no significant difference was observed between the group of 100 nM wortmannin and the control group (data not shown). From these results, we hypothesized that fusion was necessary for the transfection ability of PS-CG-HVJ-E, which was enhanced by endocytotic uptake.

Evaluation of the *in vitro* transfection efficiency of anionic liposome with or without HVJ, conjugated to cationized gelatin

To confirm this hypothesis, both anionic and HVJ-anionic liposomes were combined with cationized gelatin and protamine sulfate. When anionic liposomes without fusion protein were combined with protamine sulfate or cationized gelatin, the transfection efficiency increased compared with that of liposomes alone (Figure 4A). The combination of cationized gelatin–liposomes with protamine sulfate further enhanced transfection efficiency. A similar enhancement of transfection by protamine sulfate and cationized gelatin was seen in HVJ–liposomes (anionic liposomes with fusion proteins) (Figure 4B). However, the absolute value of luciferase gene expression by protamine sulfate–cationized gelatin–HVJ-liposomes was approximately 20 times higher than that by protamine

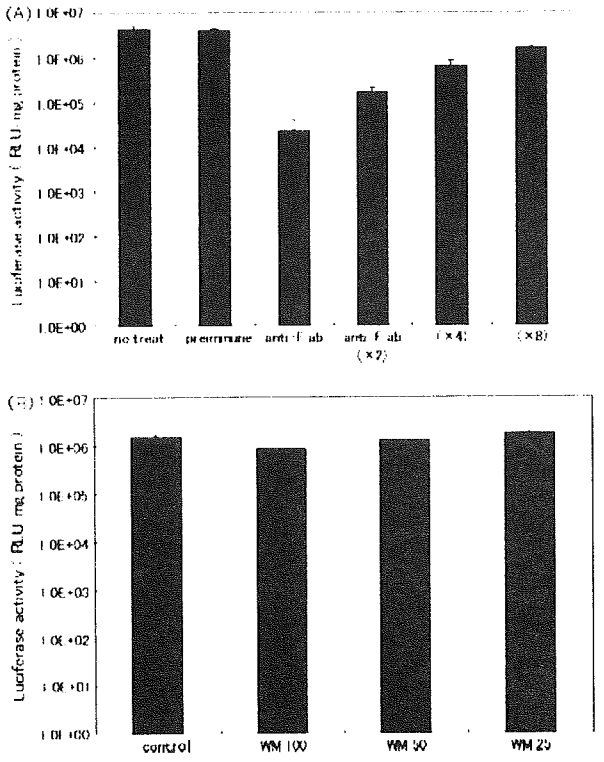

Figure 3. Effects of anti-F protein antibody (anti-F ab) (A) and wortmannin (WM) (B) on gene expression by PS-CG-HVJ-E. (A) After incubation of PS-CG-HVJ-E with antiserum, the mixture was added to CT26 cells and incubated for 10 min. Luciferase activity was measured 24 h after the removal of the mixture. Preimmune rabbit serum was used as a control. (B) CT26 cells were pretreated with various concentrations of wortmannin for 15 min. Then, the cells were subjected to gene transfer with PS-CG-HVJ-E. Luciferase activity was measured 24 h after transfer. Results are shown as mean ± s.d. (n = 3). Similar results were obtained in three independent experiments

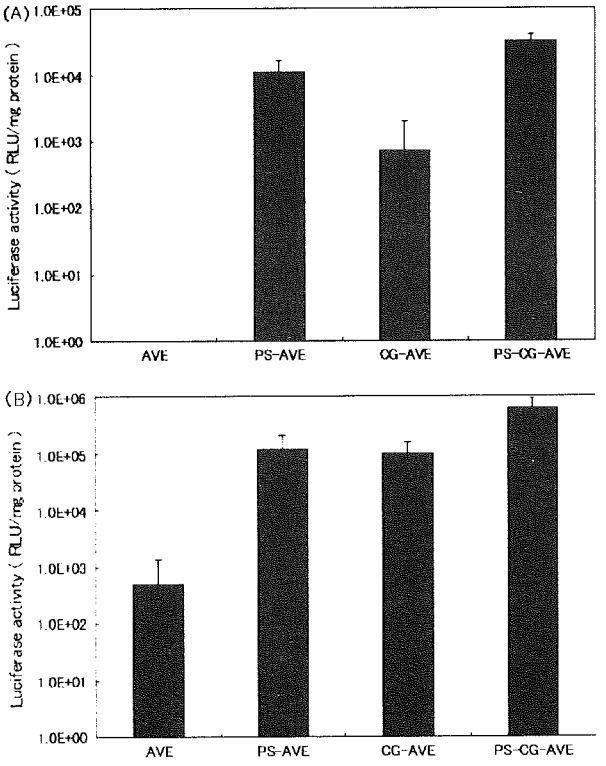

Figure 4. The effect of protamine sulfate, cationized gelatin or both on transfection efficiency by anionic liposomes (A) and anionic liposomes fused with HVJ (B). Vectors were incubated with CT26 cells for 1 h, and the luciferase activity was assessed after 24 h. AVE means anionic liposome with the same lipid components as the HIV envelope [51]. Results are shown as mean ± s.d. (n = 3). Similar results were obtained in three independent experiments

sulfate–cationized gelatin–liposomes without HVJ. Thus, gene transfer by PS-CG-HVJ-E appeared to be mediated by fusion and enhanced by endocytosis.

Specific localization of cationized gelatin-conjugated HVJ-E via intravenous administration

Next, the effect of polymer conjugation with HVJ-E on gene transfection *in vivo* was investigated (Figure 5). When HVJ-E alone was intravenously injected into the mouse tail vein, gene expression was mainly detected in the spleen. However, the gene expression was low. To enhance gene expression, HVJ-E combined with either protamine sulfate or cationized gelatin was injected into the mouse tail vein. Conjugation with protamine sulfate slightly increased luciferase expression in the liver, spleen and lung. However, CG-HVJ-E specifically enhanced gene expression in the liver approximately 100 times more than HVJ-E alone and approximately 10 times more than PS-HVJ-E. In the lung and spleen, very low levels of gene expression were observed, but no expression was detected in other organs, such as the kidney and heart. In this case, injection of PS-CG-HVJ-E resulted in lower luciferase gene expression in liver than injection of CG-HVJ-E.

Assessment of the stability of HVJ-E conjugated to cationized gelatin mixed with mouse fresh serum in comparison with HVJ-E alone

Finally, to clarify the role of cationized gelatin in enhanced *in vivo* gene transfection efficiency, CG-HVJ-E containing the luciferase gene was added to cultured cells to assess transfection efficiency after incubation with fresh mouse serum for 5 min. The transfection efficiency of HVJ-E was attenuated by incubation with mouse serum. Luciferase gene expression after the incubation of HVJ-E with fresh mouse serum at 37 °C decreased to 20% of the luciferase gene expression in the absence of mouse serum. On the other hand, luciferase gene expression after the incubation of PS-HVJ-E, CG-HVJ-E and PS-CG-HVJ-E with fresh mouse serum at 37 °C was 52.9, 72.5 and 56.7%, respectively, of the luciferase gene expression in the absence of mouse serum (Figure 6). CG-HVJ-E was

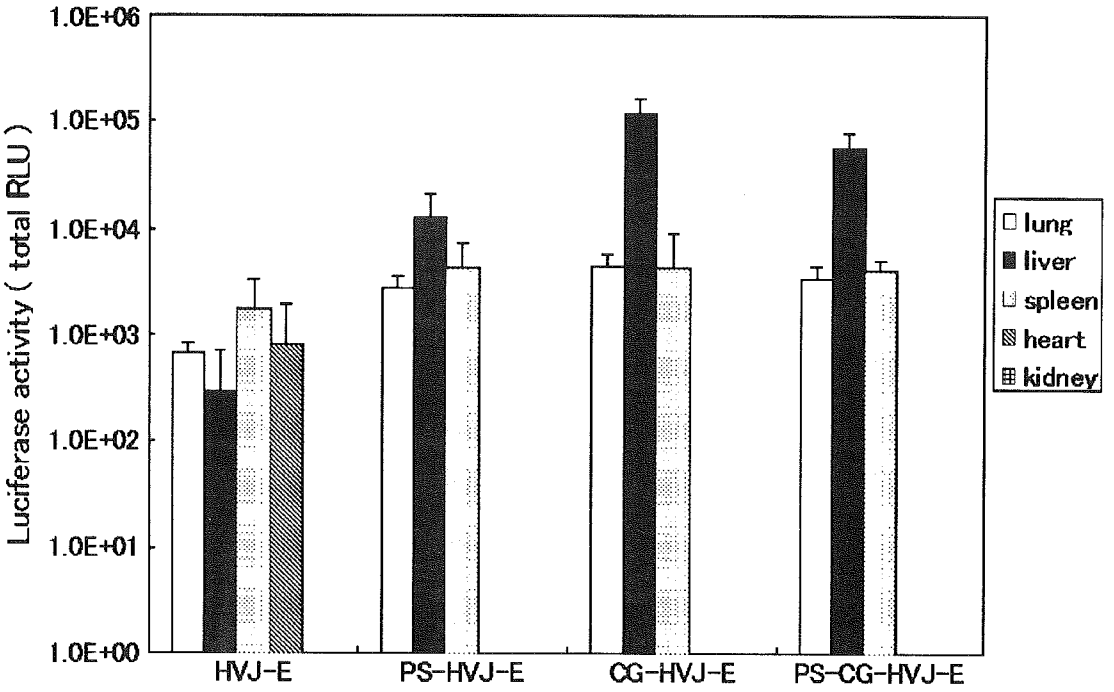

Figure 5. *In vivo* gene transfection efficiency of HVJ-E, PS-HVJ-E, CG-HVJ-E and PS-CG-HVJ-E after injection into mouse tail vein. Luciferase activity was measured in organ lysates 24 h after injection and the results are expressed as mean ± s.d. of luciferase activity of each organ from 5 to 6 mice. The group of CG-HVJ-E showed significantly higher gene expression in liver than all other groups ($P < 0.05$). Similar results were obtained in four independent experiments

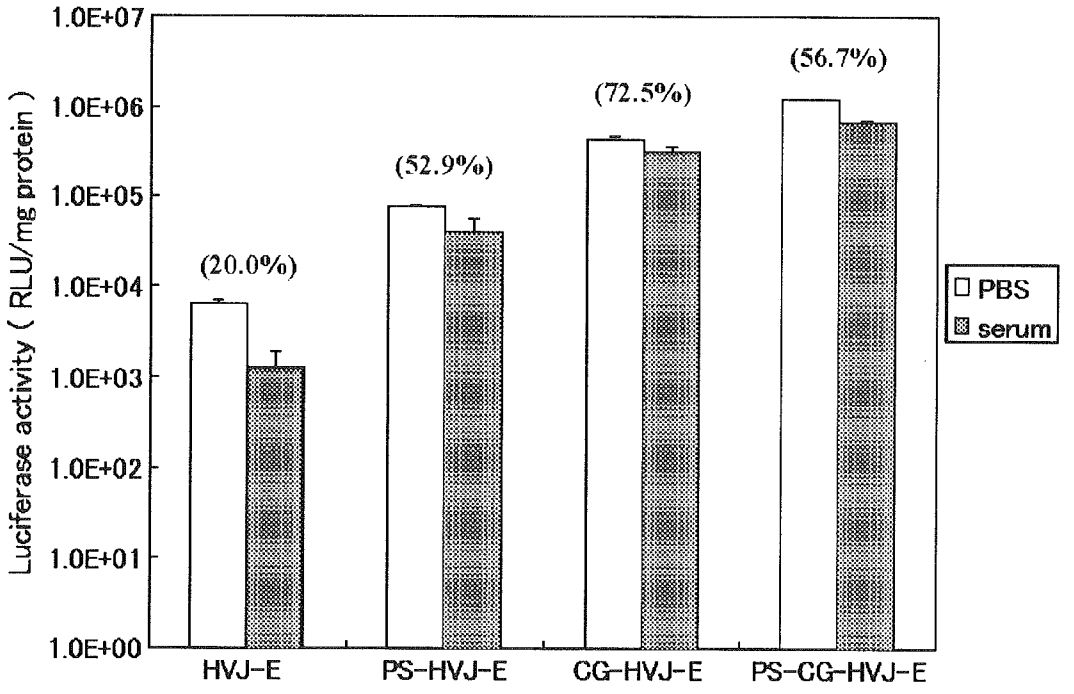

Figure 6. The effect of fresh serum on the transfection efficiency of HVJ-E or polymer-conjugated HVJ-E. After incubation of HVJ-E or polymer-conjugated-HVJ-E with fresh mouse serum, the serum was removed by centrifugation and added to CT26 cells. Luciferase activity was measured 24 h after removal of the vector. The percentage indicates the ratio of luciferase gene expression after incubation with serum (n = 3) to the luciferase gene expression after incubation with PBS (n = 3). Results are shown as mean ± s.d., respectively. Similar results were obtained in three independent experiments

the most resistant to mouse serum. Thus, we succeeded in developing a serum-resistant vector system.

Discussion

We succeeded in enhancing the transfection efficiency of HVJ-E by combining it with cationic polymers. For cultured cells *in vitro*, the most efficient transfection was obtained by combining HVJ-E with both cationized gelatin and protamine sulfate. However, for *in vivo* transfection, CG-HVJ-E without protamine sulfate resulted in the highest gene expression. These findings are consistent with our previous report indicating that the particle size of cationic liposomes may affect gene transfection efficiency [36]. By adding both protamine sulfate [37] and cationized gelatin to HVJ-E, the size and charge of the resulting complex may have been the most suitable for *in vitro* transfection. Protamine sulfate and cationized gelatin affected gene transfection efficiency in a variety of cell lines as well as in primary cells, although the efficiency was varied among cell types. The ratio of protamine sulfate and cationized gelatin used for these experiments was determined by gene transfection experiments with CT26 cells. Thus, gene expression in the other cell types may be enhanced when the conditions are optimized for each cell type.

We determined that cell fusion is the mechanism responsible for a PS-CG-HVJ-E-mediated gene transfer system. Although endocytosis appeared to be involved in gene transfection based on the wortmannin experiments, transfection was completely inhibited by antibody against the fusion protein of HVJ. Since the fusion activity of HVJ is pH-independent [31], HVJ can fuse with the cell membrane both on the cell surface and in endocytotic vesicles. Even for the HVJ-E complex with protamine sulfate and cationized gelatin, the F protein of HVJ appeared to associate with the cell membrane, and fusion activity appeared to be necessary for gene transfection.

As shown in Figure 5, HVJ-E complexed with cationized gelatin targeted the liver. With protamine sulfate, gene expression in the liver after intravenous injection was lower than with CG-HVJ-E. We speculate that larger particles with positive charge are less mobile when intravenously administered. Comparison with PS-HVJ-E and PS-CG-HVJ-E suggests that CG-HVJ-E may have the appropriate size and potential for targeting the liver after intravenous injection.

Numerous biocompatible polymers have been developed to enhance gene delivery systems [38–45]. Pullulan complexed with naked DNA targets the liver [46,47]. However, pullulan–HVJ-E complexes failed to transfect tissues, including the liver. Dextran–HVJ-E was also not an efficient complex for gene transfer. Only low molecular weight cationized gelatin has formed an effective complex with HVJ-E that enhances transfection efficiency both *in vitro* and *in vivo*, although the precise mechanism is still unknown.

Our results suggest that the CG-HVJ-E vector may be effective and practical for the treatment of liver diseases, such as liver cirrhosis and hepatitis, when therapeutic genes encoding secreted proteins, such as HGF, soluble TGF-β receptor and decorin, are employed. Moreover, long-term gene expression in the liver can be achieved with Epstein-Barr virus replicon plasmid [33] and the Sleeping Beauty transposon system [48]. CG-HVJ-E may be clinically tested in the near future because it does not require a large volume of solution to be injected (as used in the hydrodynamic_method) [48,49]. An adverse effect of this treatment is that coagulation function is transiently decreased by CG-HVJ-E in mice, although it recovered in 1 day (H. Mima and Y. Kaneda, unpubl. obs.). This adverse effect is probably caused by HVJ hemagglutinating protein, which is necessary for binding with sialic acid, a virus receptor [32]. When HVJ-E is complexed with cationized gelatin, cationized gelatin may perform the function of hemagglutinating protein and enhance the association with cell membranes. If HVJ-E without hemagglutinating protein is combined with cationized gelatin, the complex may reduce adverse effects to a much lower level.

An additional advantage of cationized gelatin is that it protects HVJ-E from degradation in fresh mouse serum. Although the *in vitro* transfection efficiency of HVJ-E was not inhibited by culture medium containing 10% FBS [32], the activity of HVJ-E was rapidly lost in the presence of fresh mouse serum (Figure 6). However, CG-HVJ-E was significantly stable in 50% fresh mouse serum. The high transfection activity of CG-HVJ-E after intravenous injection appears to be mediated by the stability of the vector in fresh serum. Retrovirus [50] and HIV [51] are degraded in human serum due to complement lysis. Liposomes composed of hydrogenated egg phosphatidylcholine and cholesterol activate the complement system in rats by interacting with IgG and IgM [52]. Although it is unproven that HVJ is degraded by complement lysis in mouse serum, the interaction of serum proteins with HVJ-E may be involved in the loss of transfection activity of HVJ-E. Conjugation to cationized gelatin appears to protect the surface molecules of HVJ-E from the detrimental effects of serum proteins.

The results of this study suggest that low molecular weight cationized gelatin may be appropriate for complex formation with various envelope viruses, such as retrovirus, herpes virus and HIV, and that the cationized gelatin–envelope virus vector may enhance transfection efficiency both *in vitro* and *in vivo*. This technology may lead to the achievement of an ideal vector system with high efficiency and minimal toxicity.

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Virus reactivation and intravenous immunoglobulin (IVIG) therapy of drug-induced hypersensitivity syndrome

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Abstract

Drug-induced hypersensitivity syndrome (DIHS) is a severe multi-organ system reaction caused by specific drugs. Many reports have revealed that human herpesvirus 6 (HHV-6) reactivation contributes to the development of DIHS. In addition, recent articles have shown that reactivation of other herpesviruses such as human herpesvirus 7 (HHV-7), Epstein-Barr virus (EBV), cytomegalovirus (CMV) might be also implicated in the development of DIHS. These observations suggest that not only HHV-6 but also other herpesvirses might reactivate from the latency and play an important role in the appearance of clinical manifestations of DIHS. Several patients with DIHS were treated with intravenous immunoglobulin (IVIG) in addition to systemic corticosteroids. The results have been encouraging although virus reactivation could not be suppressed. Although the pathomechanism of IVIG treatment in patients with DIHS remains unknown, the therapeutic effects of IVIG could be dependent, in part, on functional capabilities of anti-virus IgG contained in IVIG.

Keywords: Drug-induced hypersensitivity syndrome; Human herpesvirus 6; Virus reactivation; Intravenous immunoglobulin

1. Introduction

Drug-induced hypersensitivity syndrome (DIHS) (drug reaction with eosinophilia and systemic symptoms: DRESS) is a severe multi-organ system reaction caused by specific drugs such as anticonvulsants, salazosulfapyridine, allopurinol and minocycline. Recent reports have revealed that human herpesvirus 6 (HHV-6) reactivation contributes to the development of DIHS (Descamps et al., 1997; Suzuki et al., 1998; Tohyama et al., 1998). However, the precise mechanism by which latent HHV-6 that appears to be only reactivated under immunosuppressive conditions would reactivate in the course of DIHS remains unknown. Recently we have provided evidence that DIHS could result when long-term use of these specific drugs causes a decrease in serum immunoglobulin and circulating B cells in susceptible patients, probably via HHV-6 reactivation (Kano et al., 2004). However, it is still unknown whether HHV-6 alone reactivates from the latency in

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Table 1
Alterations of viral antibody titers

Virus	Date						
	26 August	17 September	1 October	16 October	31 October	6 November	5 December
HSV IgG (EIA)	ND	125.0	126.0	73.3	68.0	81.8	146.0
VZV IgM (EIA)	0.50	0.07	ND	ND	ND	ND	ND
VZV IgG (EIA)	25.2	74.9	ND	ND	ND	20.8	18.2
EBV VCA IgG (FA)	80	160	320	ND	ND	160	80
CMV IgM (EIA)	0.21	0.27	ND	ND	ND	1.3	ND
CMV IgG (EIA)	58.4	105.0	116.0	ND	ND	380.0	333.0
HHV-6 IgM (FA)	ND	ND	ND	ND	ND	ND	ND
HHV-6 IgG (FA)	40	5120	2560	1280	320	1280	640
			⟷ IVIG				

Abbreviations: EIA, enzyme immunoassay; FA, fluorescent antibody; ND, not determined; IVIG, intravenous immunoglobulin; HSV, herpes simplex virus; VZV, varicella-zoster virus; EBV, Epstein–Barr virus; CMV, cytomegalovirus; HHV-6, human herpesvirus 6.

the course of DIHS despite the existence of various kinds of latent viruses.

Recent articles have shown that reactivation of other herpesviruses besides HHV-6 might be also involved in the development of DIHS (Aihara et al., 2001; Descamps et al., 2003), although HHV-6 reactivation is a reliable marker for an increased risk of developing this reaction. In fact, we have already demonstrated that reactivation of human herpesvirus 7 (HHV-7) together with HHV-6 occurred in a patient with DIHS (Suzuki et al., 1998). Cytomegalovirus (CMV) reactivation in a patient with DIHS has been observed by Aihara et al. (2001). Descamps et al. have also documented that Epstein–Barr virus (EBV) infection might be implicated in the development of DIHS (Descamps et al., 2003). These observations suggest that not only HHV-6 but also other HHV-7, EBV and CMV might reactivate from the latency and play an important role in the appearance of clinical manifestations of DIHS. This is because HHV-7, EBV and CMV along with HHV-6, belong to the herpesvirus family and commonly cause illness characterized by fever and skin rashes. In addition, it has been shown that these viruses have the capacity to reactivate under immunosuppressive condition.

In view of sequential occurrence of herpesvirus reactivation observed in immunocompromised patients after bone marrow transplantation (Maeda et al., 1999, 2000), we have already suggested that a similar cascade of reactivations could be also observed during the course of DIHS based on the observation of alterations of antibody titers against herpesviruses (Kano and Shiohara, 2004).

2. Methods

To clarify this, real-time PCR assays were performed for quantitative detection of HHV-6, -7, EBV and CMV DNA in serial blood samples of a patient with DIHS. The antibody titers against herpesviruses were examined.

3. Results

DNAs of these herpesviruses were also detected in sequential order during the course of DIHS. The detection of HHV-6 DNA preceded that of HHV-7, EBV and CMV DNA. Antibody titers against these viruses significantly increased after the detection of virus DNAs in the blood (Table 1).

4. Comments

Recently, several reports have indicated an involvement of multiple organs in DIHS such as the brain, thyroid, pancreas, heart, muscle and lung. Indeed, they have revealed that encephalitis with detection of HHV-6 DNA in the cerebrospinal fluid, transient hypothyroidism and/or myocarditis can be observed during the course of DIHS (Hashimoto et al., 2003). Considering similarities between DIHS and graft-versus-host disease (GVHD) with regard to the clinical manifestations and sequential occurrence of herpesvirus reactivation, some flare-ups even for weeks after the with-

drawal of causative drugs in DIHS could be explained by the sequential reactivation of herpesviruses including HHV-6. If sequential reactivation is a possibility, then one may expect that a higher incidence of HHV-6 reactivation detected around the third week after onset represents a secondary event that requires and follows the prior reactivation of other viruses (Kano and Shiohara, 2004). Nevertheless, it is still unknown whether these herpesviruses reactivate from the latency in obligate sequential order in DIHS as demonstrated in GVHD.

Based on these findings, it seems that intravenous immunoglobulin (IVIG) treatment in patients with DIHS might be effective. Therefore, several patients with DIHS were treated with IVIG in addition to systemic corticosteroids. The results have been encouraging although HHV-6 reactivation could not be suppressed. Although the pathomechanism of IVIG treatment in patients with DIHS remains unknown, the therapeutic effects of IVIG could be dependent, in part, on functional capabilities of anti-virus IgG contained in IVIG. Thus, in the case of administrating IVIG to patients with DIHS, it is necessary to select a batch that contains a higher concentration of antibodies. This is because each immunoglobulin batch demonstrates various antibody activities against viruses.

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Case Report

Drug-Induced Hypersensitivity Syndrome Due to Cyanamide Associated With Multiple Reactivation of Human Herpesviruses

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Drug-induced hypersensitivity syndrome (DIHS), characterized by serious adverse systemic reactions in addition to skin rash, has unknown pathogenesis. Its association with human herpesvirus (HHV), mainly HHV-6, has been reported recently. A 46-year-old Japanese man is described in whom a generalized eruption developed about 1 month after taking cyanamide, a drug for alcoholism. This was associated with the following manifestations: high fever, lymphadenopathy, facial edema, marked leukocytosis with eosinophilia and atypical lymphocytes, lymphocytopenia, liver and renal dysfunction, and low IgG level. He was treated with 8 mg betamethasone daily and his condition improved, but he needed low-dose corticosteroid for almost 1 year because of several episodes of recurrence. HHV-6, HHV-7, herpes simplex virus (HSV), and cytomegalovirus (CMV) specific IgG titers showed more than a four-fold rise sequentially. Significant numbers of copies of HHV-6 and HHV-7 DNA were detected in the peripheral white blood cells by real-time polymerase chain reaction (PCR). HHV-6 and CMV DNA were detected in the serum by nested PCR. A patch test for cyanamide was positive. The diagnosis of DIHS due to cyanamide, which has never been reported as a causal drug of DIHS, accompanied by reactivation of not only HHV-6, but also HHV-7, CMV, and HSV, was made. Disturbance of the immune system was suggested by the persistent low level of IgG, and consecutive viral reactivation may have participated in the prolonged course in this case. ***J. Med. Virol. 75:430–434, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: adverse drug reactions; hypogammaglobulinaemia; polymerase chain reaction; patch test; lymphocyte stimulation test

INTRODUCTION

Cyanamide is used in the treatment of alcoholism in Japan, Europe, and North America with comparatively few side-effects [Niederhofer et al., 2003]. Only a few cases of adverse skin reactions, including allergic contact dermatitis, lichen planus-like eruptions, exfoliative dermatitis [Kawana, 1997], fixed drug eruption, and Stevens–Johnson syndrome, have been reported.

Recently, it has been recognized that drug-induced hypersensitivity syndrome (DIHS), characterized by serious adverse systemic reactions in addition to a skin rash, is associated frequently with reactivation of human herpesvirus 6 (HHV-6) [Descamps et al., 1997; Suzuki et al., 1998; Tohyama et al., 1998]. Reactivation of cytomegalovirus (CMV) [Aihara et al., 2001] or Epstein–Barr virus (EBV) [Descamps et al., 2003] has also been reported. This syndrome develops within 2–6 weeks after taking the causal drug, and tends to be prolonged for 2 weeks to several months after its discontinuation. The major causal drugs reported are anticonvulsants [Callot et al., 1996; Carroll et al., 2001;

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Descamps et al., 2001; Aihara et al., 2004], allopurinol [Suzuki et al., 1998], sulfasalazine [Tohyama et al., 1998], dapsone [Callot et al., 1996; Carroll et al., 2001], minocycline [Callot et al., 1996; Carroll et al., 2001], and mexiletine chloride [Callot et al., 1996; Mitani et al., 2003]. No case induced by cyanamide has been reported. The main symptoms of DIHS are generalized exanthematous eruption, sometimes with small pustules, facial edema, high fever, systemic lymphadenopathy, leukocytosis, eosinophilia, atypical lymphocytosis, and liver dysfunction. Other systemic manifestations such as pneumonitis, myocarditis, encephalopathy, renal dysfunction, and pancreatitis may occur. These symptoms mimic some viral infections such as infectious mononucleosis. It is suggested that viral infections participate in the development of DIHS.

In this report, a case of DIHS caused by cyanamide is presented. Reactivation of not only HHV-6, but also human herpesvirus 7 (HHV-7), herpes simplex virus (HSV), and CMV was demonstrated by increases of virus-specific IgG titers and the presence of viral DNA in peripheral white blood cells and serum detected by polymerase chain reaction (PCR).

CASE REPORT

A 46-year-old Japanese man with chronic alcoholism, iron-deficiency anaemia, gastritis, and gastroduodenal ulcer noticed an eruption on his face and back 1 year before admission. He started taking 1% liquid cyanamide, disulfiram, ferrous fumarate, and lansoprazole 36 days before the first visit to our department on November 6, 2002 (day 1). He felt that the eruption had been worsening after starting these drugs. He showed slight erythema of the face and red follicular papules on the back, and received a diagnosis of seborrheic eczema and folliculitis. However, the eruption was markedly worse the next day, with marked edema and an erythropapular rash on the face, and small vesicles and pustules superimposed on diffuse erythema of the trunk (Fig. 1). Fused target-like erythema with petechiae was observed on the extremities. Redness of the pharynx, superficial lymphadenopathy, high fever, and oliguria were also recognized. He discontinued all medicines and was hospitalized on day 9. The day after admission, treatment with intravenous betamethasone and an oral diuretic agent was started.

Laboratory tests on admission to hospital included: white blood cells, 32.82×10^9/L (neutrophils, 66.4%; eosinophils, 24.8%; lymphocytes, 4%; atypical lymphocytes, 0.5%); CD4/CD8 lymphocyte ratio, 4.4 (normal range: 0.8–2.7); hemoglobin, 12.5 g/dL; platelet count, 253×10^9/L; serum total protein, 5.1 g/dL; albumin, 2.9 g/dL; alanine aminotransferase, 14 IU/L (normal range: 11–45); aspartate aminotransferase, 10 IU/L (normal range: 14–32); lactate dehydrogenase, 273 IU/L (normal range: 116–199); alkaline phosphatase, 457 IU/L (normal range: 109–312); creatinine, 1.26 mg/dL (normal range: 0.68–1.04); IgG, 572 mg/dL (normal range: 870–1,700); IgA, 129 mg/dL (normal range: 110–410); IgM, 85 mg/dL (normal range: 33–190); Ig E, 28 IU m/L (normal range: 0–300).

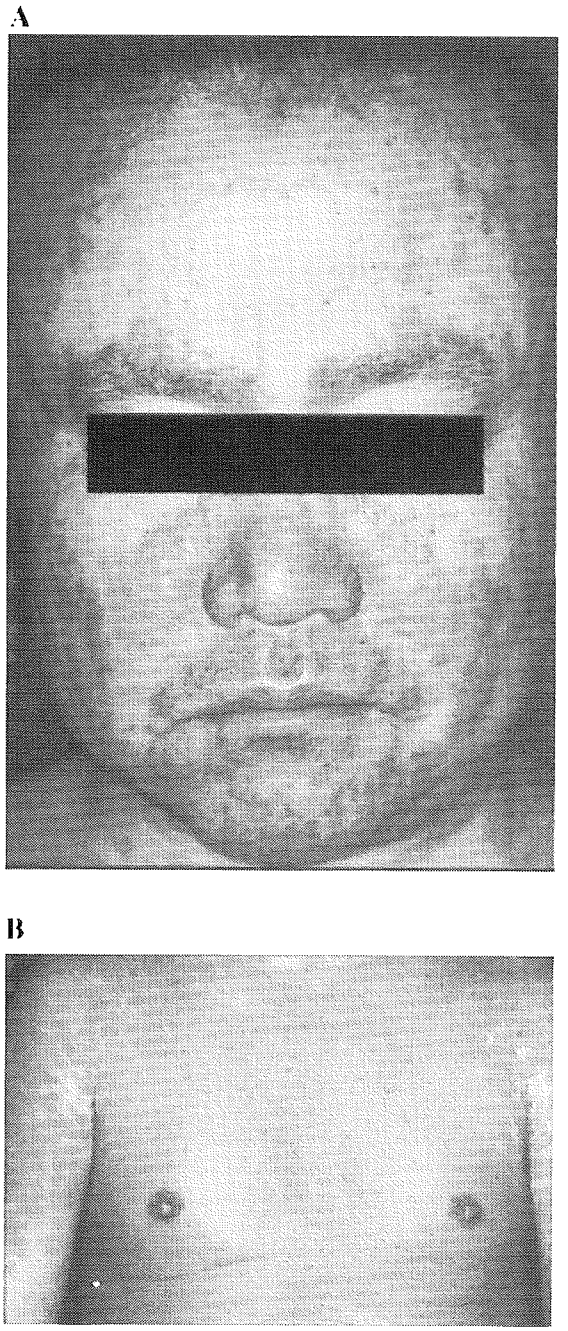

Fig. 1. Clinical features on admission. **A**: Erythropapular rash with marked facial edema. **B**: Diffuse erythema with small vesicles and pustules on the trunk.

He agreed to skin biopsy on the sixth day of systemic corticosteroid therapy. The specimen from the thigh revealed marked lymphocytic infiltration with few eosinophils in the upper dermis and around vessels of the superficial plexus. Vacuolar degeneration, lymphocytes infiltrating the dermoepidermal interface, and

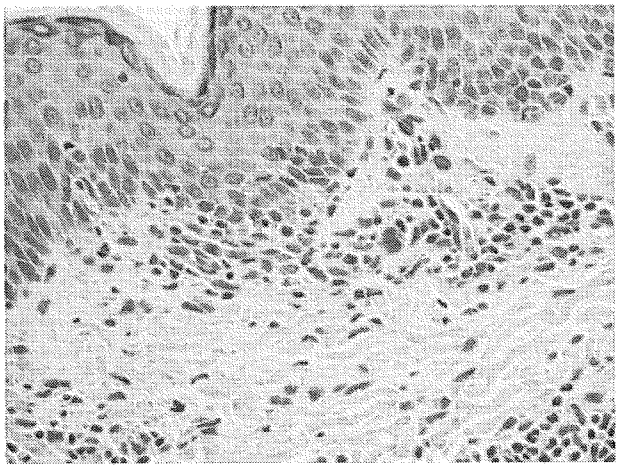

Fig. 2. Biopsy specimen revealing lichenoid tissue reaction (hematoxylin–eosin stain).

some apoptotic keratinocytes were observed, which was considered to be a lichenoid tissue reaction (Fig. 2). Immunohistochemically, CD4-positive T cells were observed more predominantly than CD8-positive T cells in the dermis and dermoepidermal interface. CD4-positive T cells were also found in the stratum spinosum. Expression of HLA-DR and ICAM-1 was found not only in infiltrating cells but also in the lower half of the epidermis. CD1a antigen (a marker of Langerhans' cells) was expressed on several cells in the basal layer of the epidermis and stratum spinosum.

Intravenous betamethasone (8 mg/day) and an oral diuretic agent were effective in improving the general symptoms, skin eruption, eosinophilia, and serum creatinine level, though white blood cells and atypical lymphocytes increased with the appearance of liver dysfunction, which improved over the following days. Corticosteroid was tapered carefully. However, red papules and slight erythema mainly on the face recurred several times, and then low-dose oral prednisolone was needed for almost 1 year. No other obvious extracutaneous symptom was accompanied except some rises of eosinophils up to near 10^9/L and low-grade liver dysfunction, which was considered due to excessive alcohol intake (Fig. 3). The low level of IgG persisted.

Patch tests for 1% liquid cyanamide and the other drugs administered were performed under treatment with 13 mg prednisolone per day, and the reaction was evaluated at 48 and 72 hr after application as recommended by the International Contact Dermatitis Research Group [Rietschel and Fowler, 2001]. The reaction to cyanamide was +++ both times, while the reactions in healthy volunteers were negative. The reactions to other drugs were also negative. Lymphocyte stimulation test for cyanamide was negative.

Virological investigations of samples of serum and peripheral white blood cells obtained serially were performed to examine viral infection. As shown in

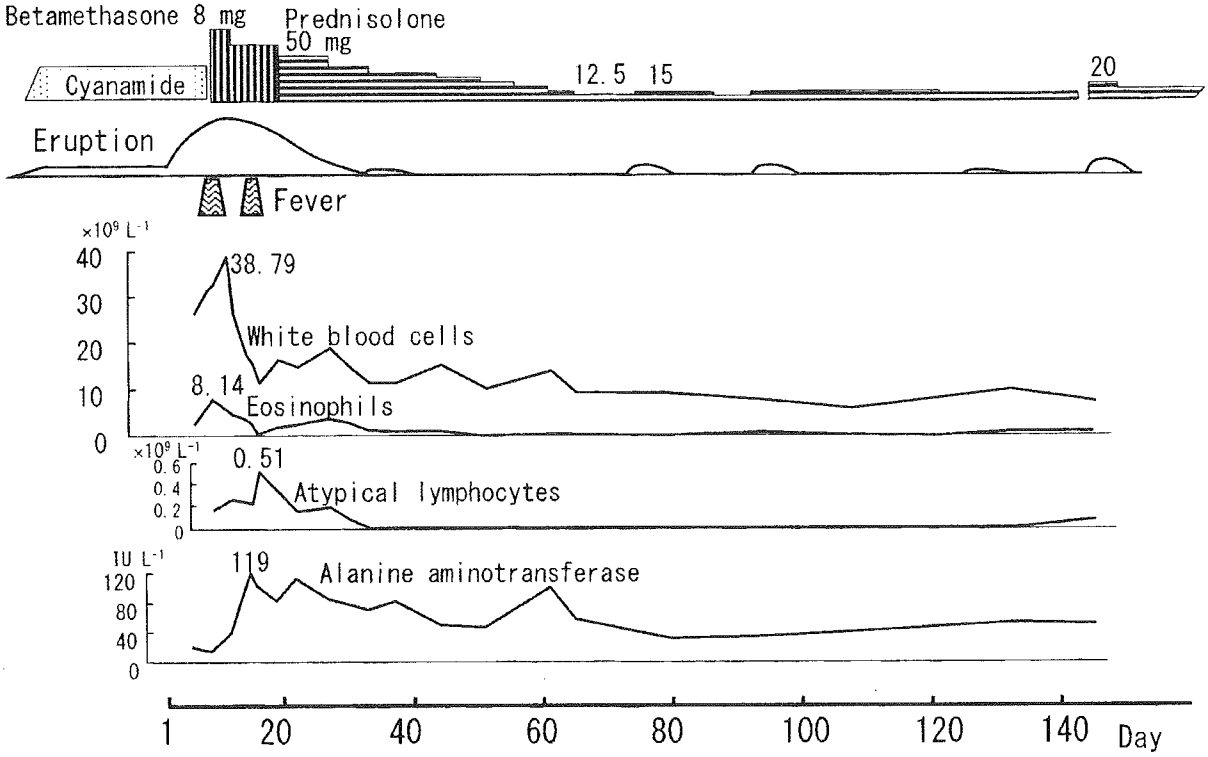

Fig. 3. Clinical course of the patient. Day 1 represents the day when the patient first attended our hospital. The eruption was markedly worse the next day.

TABLE I. Virus Specific IgG Titers

	Method	Day 7	Day 10	Day 28	Day 38	Day 66	Day 133
HSV	EIA	ND	29.6	21.7	19.9	>128	ND
VZV	EIA	ND	8.3	10.2	6.1	13.9	ND
HHV-6	FA	1:10	ND	1:2,560	1:2,560	ND	1:1,280
HHV-7	FA	1:20	ND	1:80	1:160	ND	1:80
CMV	EIA	ND	7.4	10.4	21.1	67.7	ND
EBV VCA	ELISA	ND	1.3	0.8	0.4	1.1	ND

Day 1 represents the day when the patient first attended our hospital.
The eruption was markedly worse the next day.
HSV, herpes simplex virus; VZV, varicella-zoster virus; HHV-6, human herpesvirus 6; HHV-7, human herpesvirus 7; CMV, cytomegalovirus; EBV, Epstein–Barr virus; VCA, virus capsid antigen; EIA, enzyme immunoassay. Values correspond to units/ml; FA, fluorescent antibody method; ELISA, enzyme-linked immunosorbent assay. Values correspond to units/ml.
ND, not done.

Table I, specific IgG titers for HHV-6, HHV-7, CMV, and HSV increased consecutively. Serological tests for hepatitis B virus and hepatitis C virus were negative. As shown in Table II, real-time PCR assays for HHV-6 [Tanaka et al., 2000] and HHV-7 DNA [Hara et al., 2002] in peripheral white blood cells showed significant number of copies on day 17 but not on other days. Nested PCR for HHV-6, HHV-7, and CMV DNA [Nagae et al., 1998] was also performed on serum samples, from which DNA was extracted after ultracentrifugation (10^5g, 4 hr, 4 C) to maximize sensitivity. HHV-6 DNA was detected on day 13, but not on other days. HHV-7 DNA was not detected. CMV DNA was detected on day 17, 38, and 45.

DISCUSSION

Cyanamide (H_2N-C≡N), an inhibitor of aldehyde dehydrogenase, is used as a pharmacological adjunct in the aversive treatment of chronic alcoholism. Besides other previous reports of cyanamide-induced eruption, Kawana [1997] described six cases of exfoliative dermatitis and noted that cyanamide-induced eruptions might not be rare. Few of those patients had fever, lymphadenopathy, leukocytosis, or eosinophilia, but no liver dysfunction except one, who had had liver cirrhosis before the administration of cyanamide. A relationship has not been described between viral infection and adverse reaction due to cyanamide.

The present case of DIHS caused by cyanamide was associated with reactivation of HHV-7, CMV, and HSV in addition to HHV-6. In this case, a skin eruption developed after the administration of cyanamide for 37 days, accompanied by severe systemic symptoms and low IgG level. Viral reactivation was confirmed by increases of virus-specific IgG titers and the presence of viral DNA in serum or peripheral white blood cells by PCR. The relation between viral reactivation and the clinical manifestations or treatment with systemic corticosteroid was unclear. However, the relapse of fever and increases of alanine aminotransferase and atypical lymphocytes were recognized in the same period when HHV-6, HHV-7, and CMV DNA were detected in white blood cells and serum. Lymphocytopenia may have been related to HHV-6, as in a patient described previously with a fatal CMV infection due to severe and prolonged lymphocyte depletion associated with HHV-6 reactivation [Yoshikawa et al., 2002].

HHV-6, HHV-7, and CMV, which belong to the human beta-herpesviruses, infect many healthy adults latently, like other human herpesviruses, and are reactivated especially in immunocompromised patients such as organ transplant recipients. On the other hand, the immunomodulatory effects of beta-herpesviruses have been shown in vivo and in vitro: HHV-6 depletes CD4 T lymphocytes, downregulates CD3 and CD46 transcription, alters expression of cytokines and receptors, decreases lymphocyte proliferation, and upregulates cytotoxicity of natural killer cells, while CMV impairs the ability of lymphocytes and monocytes to produce and respond to cytokines, and impairs the function of antigen-specific cytotoxic T cells and natural killer cells [Boeckh and Nichols, 2003; Dockrell, 2003].

The mechanism of viral reactivation in DIHS has not been elucidated. In this patient, the persistent low level of IgG might have contributed to multiple reactivation of herpesviruses, which in turn might have contributed to the severity of the disease, prolongation, or recurrence of

TABLE II. PCR Analysis for HHV-6, HHV-7, and CMV

	Samples	Method	Day 10	Day 13	Day 17	Day 20	Day 31	Day 38	Day 45	Day 53
HHV-6	White blood cells	Real-time PCR[a]	ND	ND	900	20	<20	<20	<20	ND
	Serum	Nested PCR	–	+	–	ND	ND	–	–	–
HHV-7	White blood cells	Real-time PCR[a]	ND	ND	1,600	<20	<20	<20	ND	ND
	Serum	Nested PCR	–	–	–	ND	ND	–	–	–
CMV	Serum	Nested PCR	–	–	+	ND	ND	+	+	–

PCR, polymerase chain reaction.
–, not detected; +, detected.
[a]Counts of DNA copies per 10^6 cells.

symptoms in this patient. Hypogammaglobulinaemia associated with DIHS has been reported [Callot et al., 1996; Descamps et al., 2001; Aihara et al., 2003], although it is known that anticonvulsants, the major causal drugs of DIHS, sometimes induce hypogammaglobulinaemia even without symptoms of DIHS. In this patient, it seems that drugs did not induce the low level of IgG because it continued over half a year after withdrawal of the causal drugs. Excessive alcohol intake and low nourishment might have caused hypogammaglobulinaemia. Other than the low level of IgG, the decrease of peripheral lymphocytes and CD8 cells, cytotoxic T cells, or other possible immunosuppressive conditions might have contributed to viral reactivation. In addition to malnourishment, the immunomodulatory effects of herpesvirus as mentioned above might have caused or exacerbated an immunosuppressed state.

It has been reported that reactivation of HHV-6 and HHV-7 increases the risk of subsequent CMV infection after organ transplantation [Boeckh and Nichols, 2003; Dockrell, 2003]. In addition, both HHV-6 and HHV-7 are known to infect and reside in circulating CD4+ lymphocytes [Lusso et al., 1994], and HHV-7 infection has been reported to trigger reactivation of HHV-6 [Tanaka-Taya et al., 2000]. Taken together, in this patient, herpesvirus might have been reactivated one after another under an immunosuppressed condition, which was induced by many factors.

The marked reaction to the patch test for cyanamide under administration of 13 mg prednisolone per day, which was performed about 6 months after withdrawal of cyanamide, suggests that a strong allergic reaction to the drug persists even after a long time.

In immunocompromised patients, it might be possible that some drugs that have never been reported as causal drugs of DIHS, induce DIHS.

Further investigations are needed to clarify the mechanism by which herpesviruses are reactivated in patients with DIHS in relation to disturbance of immunocompetence.

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