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Flow cytometric analysis of effects of cytokines on the expression of varicella-zoster virus glycoproteins

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Summary

Varicella-zoster virus (VZV)-infected human embryonic fibroblast (HEF) cells were stained with monoclonal antibodies directed against VZV glycoprotein I, II and IV, and then labeled with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG. The cells were analyzed by flow cytometry. VZV-infected cells expressing VZV glycoproteins were clearly distinguished from uninfected cells. This method was useful for analyzing expression of VZV glycoproteins in different experimental conditions. Interferon α, β, and γ and tumor necrosis factor (TNF)-α reduced the percentage of positive cells and the mean fluorescence intensity of the cells expressing VZV glycoproteins. Interleukin(IL)-1β, IL-6 and TNF-β had little effect on the expression of VZV glycoproteins.

Varicella-zoster virus; Glycoprotein; Flow cytometry; Interferon; Tumor necrosis factor

Introduction

Varicella-zoster virus (VZV) is a human herpes virus that causes chicken pox and shingles. Cells infected with VZV express over 30 polypeptides (Shiraki et al., 1982) and several virus-specific glycoproteins. Monoclonal antibodies that

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react with VZV glycoproteins have been developed by several laboratories (Grose et al., 1983; Okuno et al., 1983; Forghani et al., 1984). Four major glycoproteins of VZV have been identified (gp I, II, III and IV) (Davison et al., 1986), and the processing of these glycoproteins has been studied (Namazue et al., 1985). These glycoproteins have biologic and immunologic functions (Keller et al., 1984; Ito et al., 1985; Grose et al., 1988).

Recently flow cytometry has been used to analyze the expression of viral proteins (Elmendorf et al., 1988). Immediate early, early and late antigens of human cytomegalovirus was detected by flow cytometry (Schols et al., 1989). Litwin et al. (1990) reported cell surface expression of VZV glycoproteins by flow cytometry. In this study, we used flow cytometry to analyze intracellular and cell surface VZV glycoproteins, and we investigated the effects of cytokines on the expression of VZV glycoproteins in VZV-infected cells.

Materials and Methods

Cells and viruses

Human embryonic fibroblast (HEF) cells were grown at 37°C in Eagle's minimal essential medium (MEM; Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY). Confluent HEF cells were maintained in MEM with 2% FBS. HEF cells were used in experiments at passage level 15–20. Kawaguchi strain of VZV was provided by Professor M. Takahashi (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). Cell-free VZV was obtained from VZV-infected HEF cells by ultrasonication in PSGC medium (phosphate-buffered saline containing 5% sucrose, 0.1% sodium glutamate and 10% fetal bovine serum). The titer of cell-free VZV was 1×10^4 PFU/ml.

Monoclonal antibodies (moAbs) against VZV glycoproteins

Monoclonal antibodies directed against VZV glycoproteins were provided by Professor K. Yamanishi (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). Monoclonal antibody (moAb) from clone 9 (IgG2a) reacts with gp I, moAb from clone 8 (IgG 2b) reacts with gp II and moAb from clone 12 (IgG2a) reacts with gp IV [3].

Cytokines

The cytokines used included recombinant human interferon-α (IFN-α) (Chemicon Institutional Inc. Temecula, CA), recombinant human IFN β (Genzyme, Cambridge, MA), recombinant human IFN-γ (Genzyme), tumor necrosis factor α (TNF-α) (Asahi Chem, Tokyo, Japan), interleukin (IL)-1 β (IL-1 β) (Genzyme), IL-6 (Genzyme) and TNF-β (Genzyme). Mouse IgG1 and IgG2a (Beckton Dickinson, San Jose, CA) were used as a control antibody.

Fixation and staining

Monolayers of HEF cells were grown in 12-well culture plates (Coster, Cambridge, MA) and infected with cell-free VZV (1000 PFU/well). The multiplicity of infection (MOI) was 0.005. After adsorption for 1 h, maintenance medium (MEM with 2% FBS) was added and HEF cells were incubated at 37°C for 3 days. The cells were washed with PBS 3 times and detached with 0.25% trypsin and 0.01% EDTA. One milliliter of PBS with 10% FBS was added to each well, and the cell suspensions were transferred to polystyrene tubes and centrifuged for 5 min at 500 × *g*. The cells were then washed in cold PBS, centrifuged, and resuspended in 90% methanol. The mixtures were kept at −20°C for 20 min. The cells were then washed once with cold PBS and resuspended in 1 ml of PBS containing 25% normal goat serum (Dakopatts, Denmark), 0.002% Triton X-100, and 0.1% sodium azide (PBS-NGS). After 20 min incubation at room temperature, the cells were centrifuged at 500 × *g* for 5 min, the supernatant was removed, and 10 μl of diluted moAb was added to the cell pellets. MoAbs were used at a final dilution of 1:100. After 1 h incubation at 37°C, the cells were washed twice with cold PBS-NGS and then incubated for 1 h at 37°C with fluorescein isothiocyanate (FITC) conjugated affinity purified F(ab')2 goat anti-mouse IgG (Cappel, West Chester, PA). The cells were washed twice with PBS-NGS and resuspended in 0.5 ml of PBS-NGS.

Flow cytometric analysis

The cells were analyzed by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA). Five thousand events per sample were collected in a logarithmic mode, stored and analyzed in a Consort 30 system (Becton Dickinson). All data are expressed in a log fluorescence histogram. Expression of VZV glycoproteins under various experimental conditions was compared by calculating the relative mean fluorescence (rMF) and the percent reduction in the presence of cytokines. Fluorescence intensity expressed by log scale was converted to a linear mode. Mean fluorescence of all gated cells was expressed as linear mean fluorescence (lMF). The rMF and percent reduction in fluorescence in the presence of cytokines were calculated using the following formulas:

$$\text{rMF} = \text{lMF (VZV infected cells)/lMF (uninfected cells)}$$

$$\% \text{ reduction} = [1 - (\text{E-U/C-U})] \times 100$$

E: lMF obtained with VZV-infected cells in the presence of cytokine;
C: lMF obtained with VZV-infected cells;
U: lMF obtained with uninfected cells.

Effects of cytokines on expression of VZV glycoproteins

When HEF cells became confluent, the medium was removed and

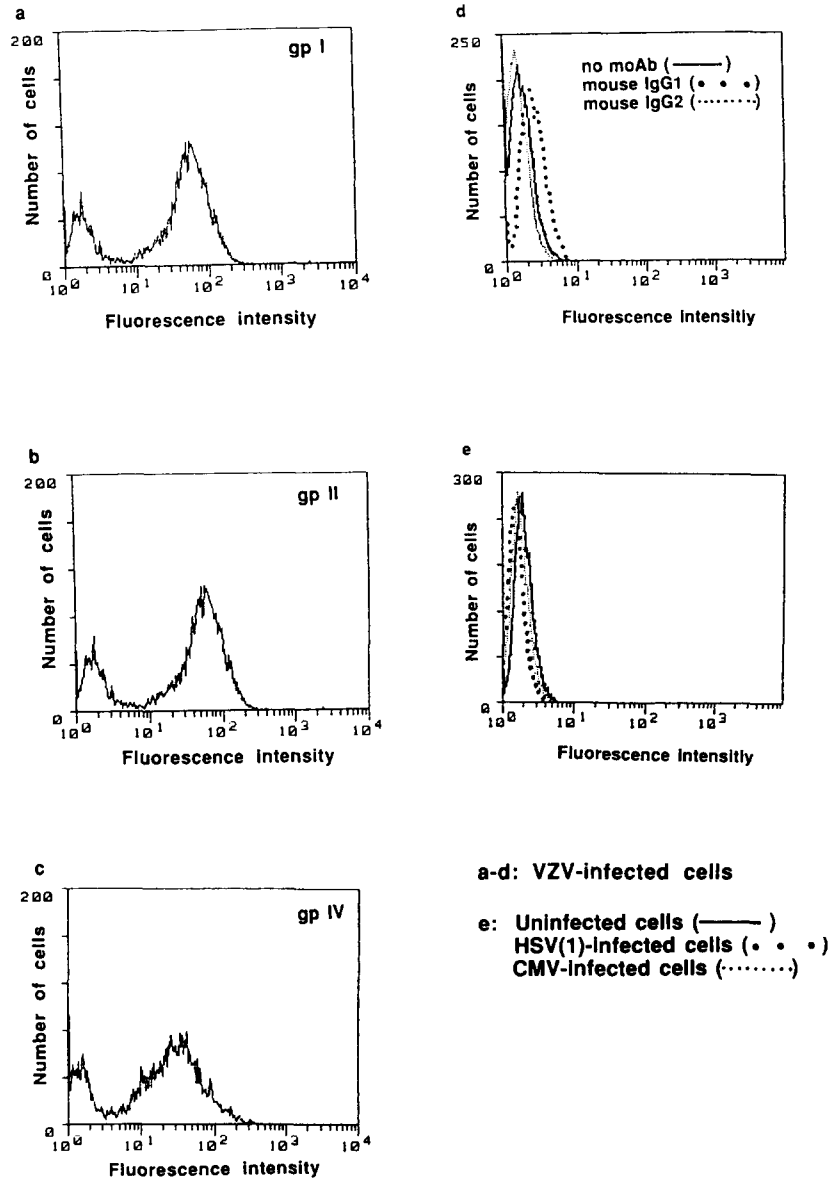

Fig. 1. Analysis of human embryonic fibroblast (HEF) cells for expression of VZV glycoproteins. a–c: VZV-infected HEF cells were stained with monoclonal antibodies (moAbs) directed against gp I (a), gp II (b) and gp IV (c) 72 h after infection. d: VZV-infected HEF cells were stained with no moAb, mouse IgG1 or IgG2a. e: uninfected HEF cells, CMV-infected HEF cells and HSV (type 1)-infected HEF cells were stained with moAb directed against gp I.

maintenance medium which contained the cytokine at each designated concentration was added. After incubation at 37°C for 24 h, HEF cells were

infected with cell-free VZV. After 3 days incubation, the HEF cells were fixed and stained with moAbs to VZV glycoprotein and analyzed by flow cytometry as described above. In another experiment, to determine the effect of cytokines on the expression of VZV glycoprotein after adsorption of VZV, confluent HEF cells were incubated for 3 days with maintenance medium in the presence of cytokines following adsorption of VZV.

Results

Analysis of glycoproteins of VZV-infected cells by flow cytometry

HEF cells expressing VZV glycoproteins were separated clearly from HEF cells expressing no VZV glycoproteins (Fig. 1). Cells with fluorescence intensity exceeding the upper threshold of unstained VZV-infected cells were considered positive. The percentage of HEF cells expressing gp I, II and IV was 67.7%, 75.1% and 76.5%, respectively. VZV-infected cells stained with control mouse IgG1 and IgG2a were negative. None of the moAbs to VZV glycoproteins reacted with uninfected, cytomegalovirus (CMV) (AD169 strain)-infected or

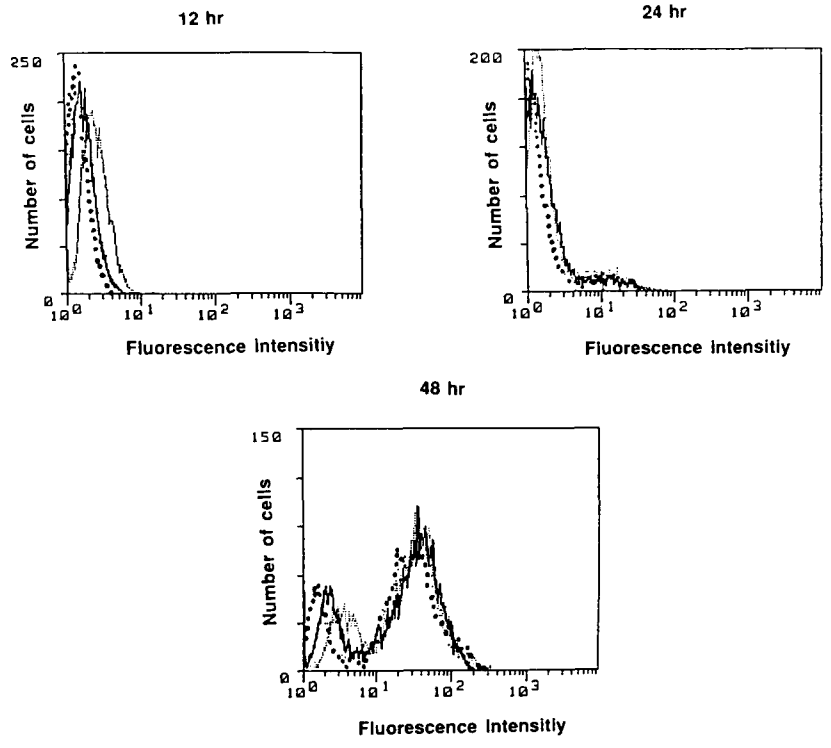

Fig. 2. Time course of expression of VZV glycoproteins. VZV-infected HEF cells were stained with monoclonal antibodies (moAbs) directed against gp I (unbroken line), gpII (heavy dotted line) and gp IV (light dotted line) 24 (left), 48 (middle) and 72 h (right) after infection.

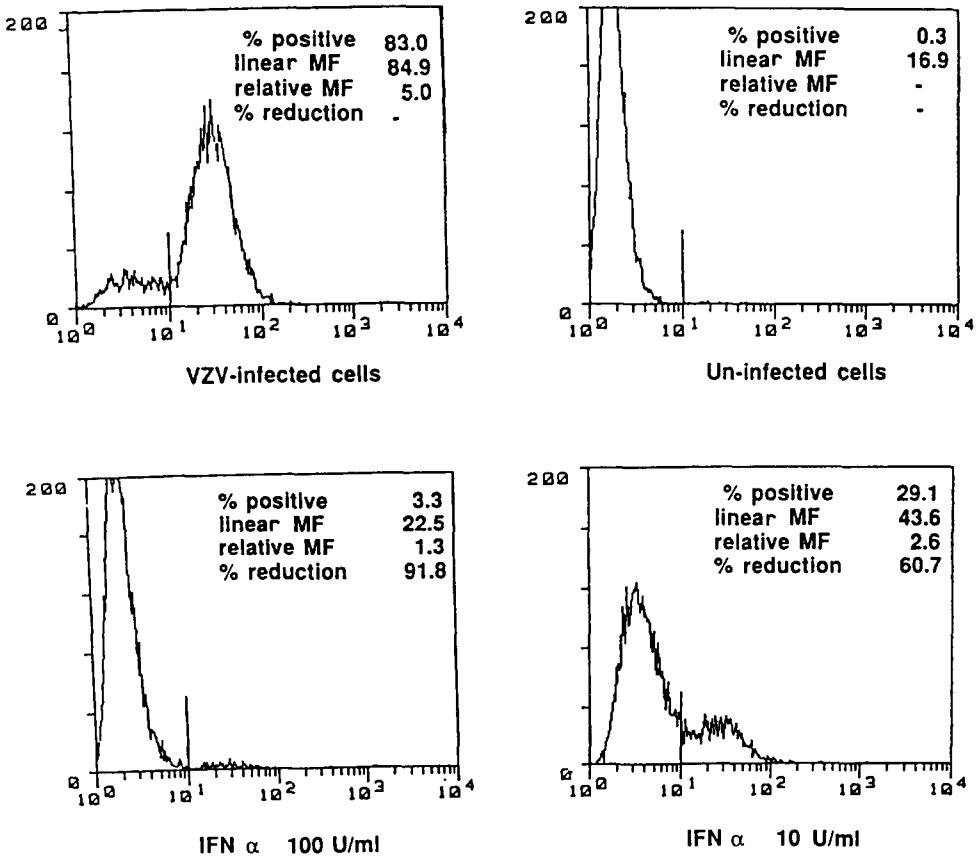

Fig. 3. Effect of IFN-α on expression of VZV gp I. HEF cells were treated with 100 U/ml and 10 U/ml of interferon α for 24 h and then infected with VZV. The cells were cultured for 3 days and then stained with moAb directed against gp I.

herpes simplex virus (HSV) (type 1, KOS strain)-infected HEF cells. When HEF cells were infected with VZV at an MOI of 0.01, the percentage of fluorescence-positive HEF cells was 80%. The percentage of HEF cells expressing VZV glycoproteins increased proportionally with MOI (data not shown).

HEF cells infected with VZV at 12, 24 and 48 h after infection at MOI of 0.005 were analyzed (Fig. 2). No VZV glycoproteins was expressed in HEF cells 12 h after infection. Expression of VZV glycoprotein increased after 48 h of infection. VZV-infected HEF cells stained with each of the moAbs showed similar histograms.

Effects of cytokines on expression of VZV glycoproteins

Monolayers of HEF cells were cultured with maintenance medium containing IFN-α, -β, -γ or TNF-α at the indicated concentration for 24 h,

TABLE 1

Effects of interferon (IFN)-α, -β, or -γ, or tumor necrosis factor (TNF) α on the expression of gp I of varicella-zoster virus (VZV)
Human embryonic fibroblast (HEF) cells were infected with VZV after treatment with IFN-α, -β, -γ, or TNF-α for 24 h, and cultured for 3 days. HEF cells were stained with monoclonal antibody directed against VZV gp I and labeled with fluorescein isothianate (FITC)-conjugated anti-mouse IgG.

		% Positive cells	lMF[†]	rMF[‡]	% Reduction
VZV-infected cells		64.2§	69.8	3.1	–
Uninfected cells		0.6	22.5	–	–
IFN-α	100 (U/ml)	7.0**	34.0**	1.6*	76.2
	10	32.3**	49.0**	2.4	43.3
	1	47.1*	58.5*	2.9	24.0
	0.1	49.2	61.1	3.4	13.5
IFN-β	100	42.3**	53.2*	1.8*	40.0
	10	55.3	62.5	2.1	20.7
	1	55.6	66.1	2.3	13.2
	0.1	57.9	67.1	3.0	10.3
IFN-γ	100	4.0**	32.7**	1.6*	79.4
	10	40.7*	54.5	1.9	37.1
	1	55.7	63.7	2.9	11.4
	0.1	62.5	64.6	3.0	7.5
TNF-α	100	13.2**	34.3**	1.2*	64.4
	10	23.6*	41.9*	1.7	49.0
	1	43.4*	56.4*	2.6	21.5
	0.1	50.7	61.3	3.1	10.9

**$P < 0.01$ (Student's t-test) compared with VZV-infected cells.
*$P < 0.05$ (Student's t-test) compared with VZV-infected cells.
[†]lMF: linear mean fluorescence;
[‡]rMF: relative mean fluorescence;
[§]Data represent mean of 4 experiments.

then HEF cells were infected with VZV and cultured for 3 days. A representative histogram of VZV-infected HEF cells treated with 100 U/ml and 10 U/ml of IFN-α is shown in Fig. 3. Percentage of VZV-infected cells expressing gp I and fluorescence intensity were clearly reduced by treatment with IFN-α. Table 1 shows the effects of IFNs and TNF-α on the expression gp I of VZV. The percentage of HEF cells expressing VZV gp I was decreased significantly when treated with IFN-α, -β, -γ or TNF-α in a dose-dependent fashion. Both the lMF and rMF of HEF cells clearly were inhibited by pretreatment with IFN-α, -β, -γ or TNF-α in a dose-dependent fashion. Effects of IFN-α, -β, -γ or TNF-α on the expression of gp II and IV were similar (data not shown).

Monolayers of HEF cells were infected with VZV then HEF cells were cultured in the presence of each cytokine. The percentage of VZV-infected cells and the fluorescence intensity was decreased by post-treatment with IFN-α, -β,

-γ or TNF-α (data not shown). However, the decrease of expression of VZV glycoproteins by post-treatment with cytokines was less than by pre-treatment with these cytokines. Monolayers of HEF cells were treated with IL-1β, IL-6 or TNF-β for 24 h, then HEF cell were infected with VZV and cultured for 3 days. The concentration of IL-1β, IL-6 and TNF-β ranged from 1 U/ml to 1000 U/ml. IL-1β and IL-6 had no effect on the expression of VZV glycoproteins. TNF-β at a high concentration (1000 U/ml) only reduced the number of cells expressing VZV glycoproteins by 15%. Expression of VZV glycoproteins were not changed when HEF cells were infected with VZV and cultured in the presence of IL-1β, IL-6 or TNF-β.

Discussion

Varicella-zoster virus contains 4 major glycoproteins designated as gp I, II, III and IV (Davison et al., 1986). Using monoclonal antibodies specific for gp I, II and IV, Okuno et al. demonstrated that these glycoproteins are present on the virions and both in the cytoplasm and on the surface of VZV-infected cells (Okuno et al., 1983).

Litwin et al. (1990) reported the membrane expression of VZV glycoproteins gp I, gp II, gp III and gp IV and cell surface expression of Fc receptor in VZV infected cells by flow cytometry. However, they did not mention intracellular glycoproteins. In this study we fixed VZV-infected HEF cells with methanol and stained VZV-infected cells with moAbs specific for gp I, II and IV. The cells were labeled with FITC-conjugated goat anti-mouse IgG and analyzed by flow cytometry. As shown in the results, VZV-infected cells expressing gp I, II and IV were distinguished clearly from uninfected cells. Both intracellular and cell surface VZV glycoproteins were detected by this procedure. These moAb did not react with CMV- and HSV-infected cells. None of control mouse IgG1, IgG2a and the moAbs to CMV antigens (IE, E and L) reacted with VZV-infected cells (data not shown). The histograms of VZV-infected cells stained with each moAb showed a similar pattern. This suggests that all three of these glycoproteins are expressed in a late phase of infection.

CMV specific antigen has been determined by flow cytometry using moAbs specific for immediate early (IE), early (E) and late (L) antigen (Elmendorf et al., 1988; Schols et al., 1989). According to previously reported methods (Jacobberger et al., 1986; Schmid et al., 1988), we calculated mean fluorescence intensity of VZV-infected cells. We consider this method useful for analyzing the expression of VZV glycoproteins because (1) several thousands of cells can be analyzed rapidly, (2) the percentage of cells expressing VZV glycoproteins and the intensity of the fluorescence of VZV infected cells can be calculated, (3) glycoproteins presence in the VZV infected cells can be shown quantitatively and (4) effects of cytokines on the expression of gp I, II and IV in different experimental conditions can be compared.

Interferon-α, -β, -γ or TNF-α have been known to inhibit replication of VZV

(Ito et al., 1991). In previous experiments we monitored the inhibitory effect of these cytokines by a plaque reduction assay (Ito et al., 1991). In this study, we demonstrated effects of cytokines on expression of VZV gp I, II and IV quantitatively by flow cytometry. Expression of gp I, II and IV was decreased clearly when pretreated with IFN-α, -β, -γ or TNF-α. Both the percentage of cells expressing VZV glycoproteins and the IMF of the cell population were inhibited by these cytokines. On the other hand, IL-1β, IL-6 and TNF β had little effect on the expression of VZV glycoproteins. The addition of IL-6 or TNF-β to IFN-α, -β, -γ or TNF-α did not affect the inhibition of VZV glycoprotein expression (data not shown).

The mechanisms of antiviral action of IFN and TNF-α have been studied (Kerr et al., 1978; Lebleu et al., 1976; Van Damme et al., 1987; Reis et al., 1989). IFNs and TNF-α may inhibit synthesis of glycoproteins, glycolysation or processing from Golgi apparatus to membrane. However, the mechanism of inhibition of expression of VZV glycoproteins by cytokines is still unknown.

This technique may also be useful for analyzing whether anti-VZV agents inhibit the expression of VZV glycoproteins. This will help to evaluate the mechanism of action of these drugs and may facilitate the development of new antiviral agents.

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