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# Co-expression of Fas and Fas-ligand on the surface of influenza virus-infected cells

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## Abstract

**Influenza virus-infected cultured cells undergo apoptosis after an increment of Fas (APO-1/CD95) on the cell surface. By flow cytometry, cell surface Fas-ligand was detected in virus-infected cells with a time course similar to that of Fas. Moreover, Fas and Fas-ligand were co-expressed in those cells. The mode of induction, however, appeared to be distinct for the two proteins. Influenza virus infection induced the externalization of phosphatidylserine on the cell surface at the early stage of apoptosis, an event that has been observed in cells undergoing Fas-mediated apoptosis. In fact, apoptosis of the virus-infected cells was inhibited in the presence of an antagonistic anti-Fas-ligand monoclonal antibody. These results suggest that influenza virus infection causes augmented expression of both Fas and Fas-ligand and apoptosis is induced when the infected cells come into contact with each other.**

**Keywords:** apoptosis induction; influenza virus; Fas; Fas-ligand; phosphatidylserine externalization

**Abbreviations:** FITC, fluorescein isothiocyanate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasolium bromide; PI, propidium iodide; PS, phosphatidylserine

## Introduction

Many viruses alter the regulation of apoptosis, either by stimulation or inhibition, in infected cells (Nakanishi, 1995; Teodoro and Branton, 1997). Clarifying the mechanism of this phenomenon could lead to the development of new therapeutics against virus diseases.

Influenza virus causes apoptotic death of cultured cell lines (Takizawa *et al*, 1993; Fesq *et al*, 1994; Hinshaw *et al*, 1994) as well as of tissues in infected animals (Mori *et al*, 1995). We previously showed that production of Fas (Peter *et al*, 1996; Nagata, 1997), also called APO-1 or CD95, increases at the transcription level in influenza virus-infected cells prior to their death (Takizawa *et al*, 1993, 1995). Since the addition of an apoptosis-antagonizing anti-Fas monoclonal antibody partly inhibited apoptosis of infected cells (Takizawa *et al*, 1995), it has been suggested that virus-induced apoptosis involves the Fas system. However, it is not clear whether the expression of Fas-ligand is also induced upon influenza virus infection. We here examined the presence of Fas-ligand on the surface of virus-infected cells using a monoclonal antibody against Fas-ligand, and the same antibody was used to influence apoptosis caused by the virus infection.

## Results

### Co-expression of Fas and Fas-ligand on the surface of influenza virus-infected cells

HeLa cells were infected with influenza virus, harvested at various time points and subjected to a flow cytometric analysis of cell surface Fas and Fas-ligand (Figure 1). In line with our previous observation (Takizawa *et al*, 1995), the amount of cell surface Fas increased at about 6–12 h after infection. Fas-ligand also became detectable on the surface of the virus-infected cells with a time course similar to that of Fas. The presence of a soluble form of Fas-ligand (Tanaka *et al*, 1995) was not likely since the addition of the cultured medium did not induce apoptosis of uninfected HeLa cells (data not shown). We next examined whether Fas and Fas-ligand are co-expressed in the virus-infected cells by doubly staining the cells with antibodies against the two proteins. As shown in Figure 2, more than 70% of the infected cells were positive for both Fas and Fas-ligand at 24 h after infection, indicating that both proteins are simultaneously expressed on the cell surface.

We previously reported that expression of the Fas-encoding gene is stimulated without apoptosis induction when HeLa cells are cultured in the presence of poly(I)-poly(C), a synthetic double-stranded RNA (Takizawa *et al*, 1995). We examined whether the same effect is observed for Fas-ligand. Expression of cell surface Fas increased on the addition of poly(I)-poly(C) whereas that of Fas-ligand changed little (Figure 3). In addition, HeLa cells showed a basal level expression of Fas as previously observed, but this was not the case with Fas-ligand. These results explained why poly(I)-poly(C)-treated cells do not undergo apoptosis irrespective of an increment of Fas production, and indicated that the regulatory mechanisms underlying expression of Fas and Fas-ligand are distinct from each other.

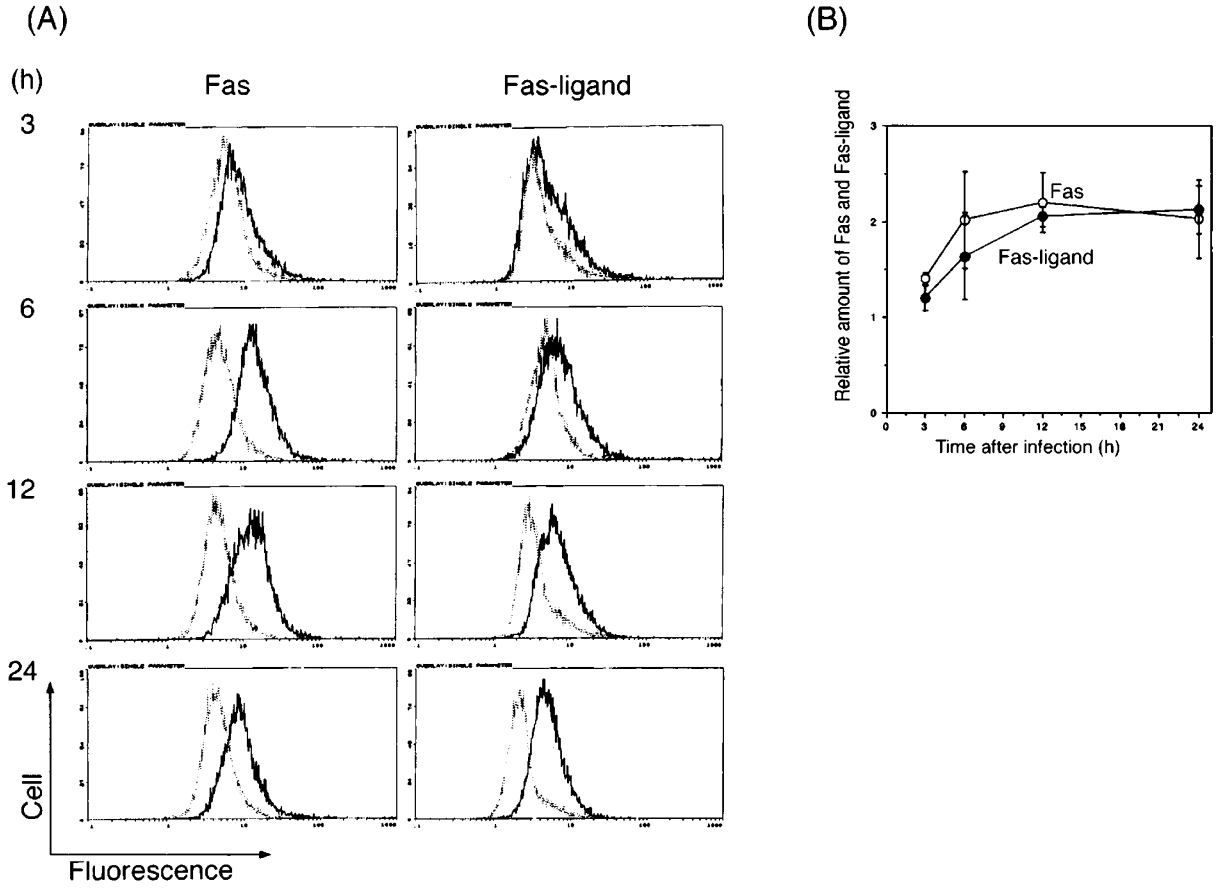


**Figure 1** Cell surface expression of Fas and Fas-ligand upon influenza virus infection. HeLa cells were infected with the virus and examined for the presence of cell surface Fas and Fas-ligand by flow cytometry. (**A**) The virus-infected (solid lines) and mock (broken lines) cells were treated with an anti-human Fas antibody or an anti-human Fas-ligand antibody, and the cells bound by a primary antibody were detected with a FITC-conjugated secondary antibody. (**B**) Semi-quantitative representation of the amount of Fas (open circles) and Fas-ligand (closed circles) on the surface of the virus-infected cells. The mean fluorescence in the virus-infected cells was divided by that in mock cells, and the mean and standard deviations from three independent experiments are shown

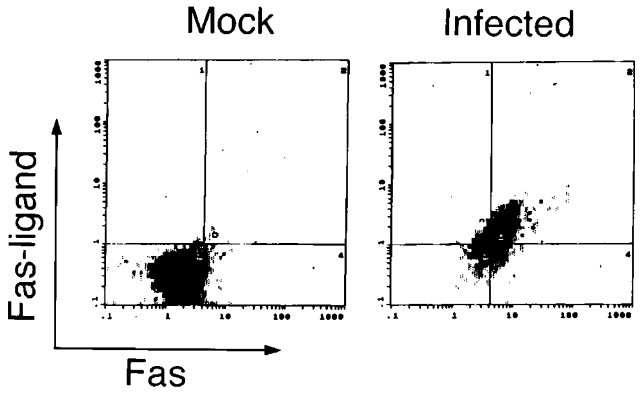


**Figure 2** Co-expression of Fas and Fas-ligand in influenza virus-infected cells. HeLa cells infected with the virus for 24 h were subjected to a two-color analysis of Fas and Fas-ligand on the cell surface. Shown is a result from one experiment of many similar results

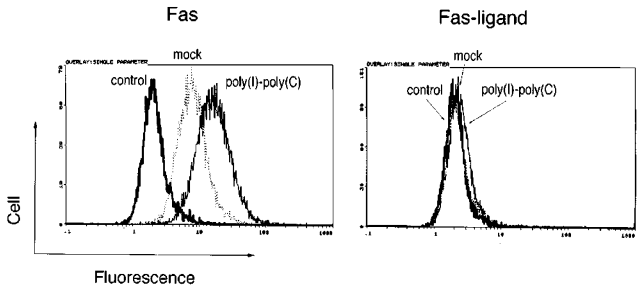


**Figure 3** Effect of a double-stranded RNA on expression of Fas and Fas-ligand. HeLa cells were treated with poly(I)-poly(C) or a buffer alone (control, mock) and subjected to flow cytometric analyses of cell surface Fas and Fas-ligand, control, cells analyzed with no primary antibodies

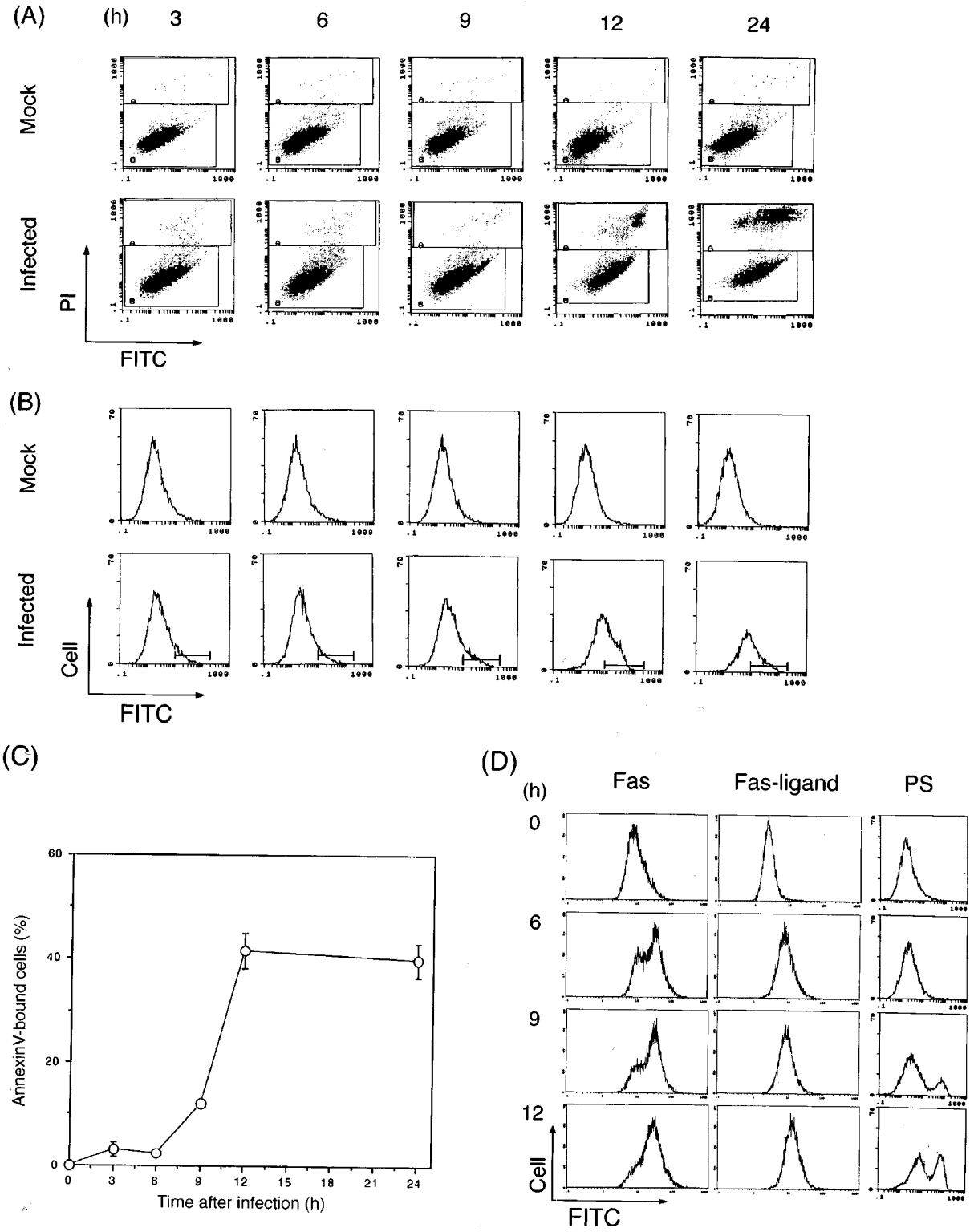


**Figure 4** PS externalization in influenza virus-infected cells. The virus-infected and mock cells were treated with PI and FITC-labeled annexin V, and the presence of PS on the cell surface was determined by flow cytometry. (**A**) Signals from PI and FITC were determined. (**B**) The PI-negative cells were analyzed for the binding of annexin V. (**C**) The relative number of cells with more FITC signals, indicated with horizontal bars in (**B**), is shown with the mean and standard deviations from three independent experiments. (**D**) Simultaneous evaluation of Fas and Fas-ligand expression and PS externalization. Note that different virus stocks were used in (**B**) and (**D**)

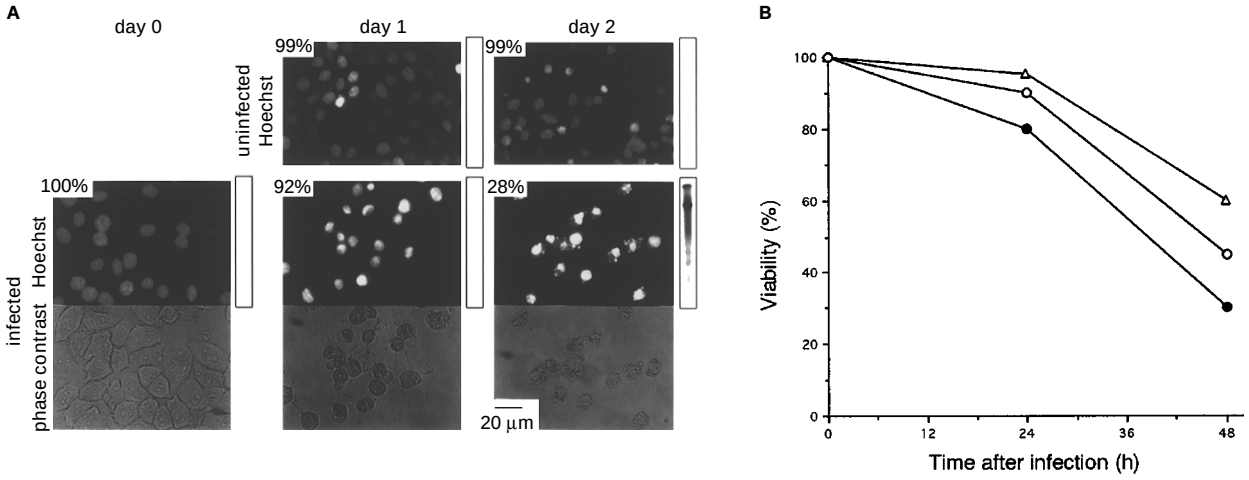


**Figure 5** Apoptotic changes upon influenza virus infection. (**A**) Morphology, chromatin condensation, membrane permeability and DNA fragmentation in influenza virus-infected HeLa cells were determined. The cells were stained with Hoechst 33342 and examined by fluorescence (upper five panels) and phase-contrast (lower three panels) microscopy. The same cell populations were examined for membrane permeability by a trypan blue exclusion test and for DNA fragmentation; percentages of the cells with intact cell membranes are indicated in the inlets and autoradiograms of agarose gels are shown at the right of each panel. (**B**) Integrity of mitochondria and membrane permeability were determined. Cell viability in terms of trypan blue exclusion (○), MTT conversion (△), and $^{51}Cr$ release (●) is shown as the average from two independent experiments

### Externalization of phosphatidylserine on the surface of influenza virus-infected cells

The above results suggested that apoptotic death of the influenza virus-infected cells is mediated by Fas and Fas-ligand. Cells undergoing Fas-mediated apoptosis show externalization of phosphatidylserine (PS) (Martin *et al*, 1995, 1996b; Conrad Liles *et al*, 1996), a phospholipid which is normally confined to the cytoplasmic leaflet of the membrane bilayer (Zachowski, 1993; Zwaal and Schroit, 1997), prior to other changes observed in apoptotic cells (Martin *et al*, 1995). To examine the occurrence of PS externalization upon influenza virus infection, cells were treated with fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide (PI), and analyzed in a flow cytometer. We observed two distinct cell populations of virus-infected cells in terms of the PI positivity (Figure 4A); it was presumed that the cells with less staining possessed intact plasma membranes, while the membrane of cells that were more intensely stained with PI were damaged. The ratio of PI-positive cells increased as the culture period was prolonged, indicating the occurrence of cell death. The cells that were weakly stained with PI were examined for the binding of annexin V (Figure 4B). The fluorescence intensity of a major peak began to shift at 9 h after infection, and the change was completed by 12 h (Figure 4C). These results indicated that PS externalization occurred synchronously in the influenza virus-infected cells after the induction of Fas and Fas-ligand (Figure 4D).

Other apoptotic changes such as chromatin condensation, DNA ladder formation, an increase of membrane permeability and inactivation of mitochondrial enzymes, all became evident at 24 h and later after infection (Figure 5). These results indicated that PS externalization precedes other apoptotic events examined so far. Influenza virus-infected cells thus seemingly behave like cells undergoing Fas-mediated apoptosis.

### Inhibition of apoptotic death of influenza virus-infected cells by antagonistic anti-Fas-ligand antibody

We previously showed that the addition of anti-Fas monoclonal antibody clone ZB4 only partially inhibited apoptosis of influenza virus-infected cells (Takizawa *et al*, 1995). To further examine the involvement of Fas and Fas-ligand in the death of virus-infected cells, HeLa cells were infected with influenza virus in the presence of an anti-Fas-ligand monoclonal antibody, 4H9, that neutralizes the activity of Fas-ligand (Tanaka *et al*, 1996). The extent of cell death was determined in terms of PS externalization (Figure 6A) and permeabilization of the plasma membrane (Figure 6B). The addition of the antibody brought about a marked inhibition of both early and later apoptotic events caused by influenza virus infection, whereas the same amount of control IgG did not show any effect. Some apoptotic virus-infected cells were resistant to the Fas-ligand-antagonizing antibody, suggesting the occurrence of Fas-independent apoptosis. These results indicated that apoptosis of the influenza virus-infected cells is caused, though not completely, by Fas and Fas-ligand.

## Discussion

We propose the following mechanism for apoptosis induction in influenza virus-infected cells; the virus-infected cells possess both Fas and Fas-ligand on the surface and apoptosis is induced when such cells attach to each other bringing about the interaction of Fas-ligand with its receptor Fas. Evidence has been accumulating that some stimuli activate the production of Fas-ligand which subsequently

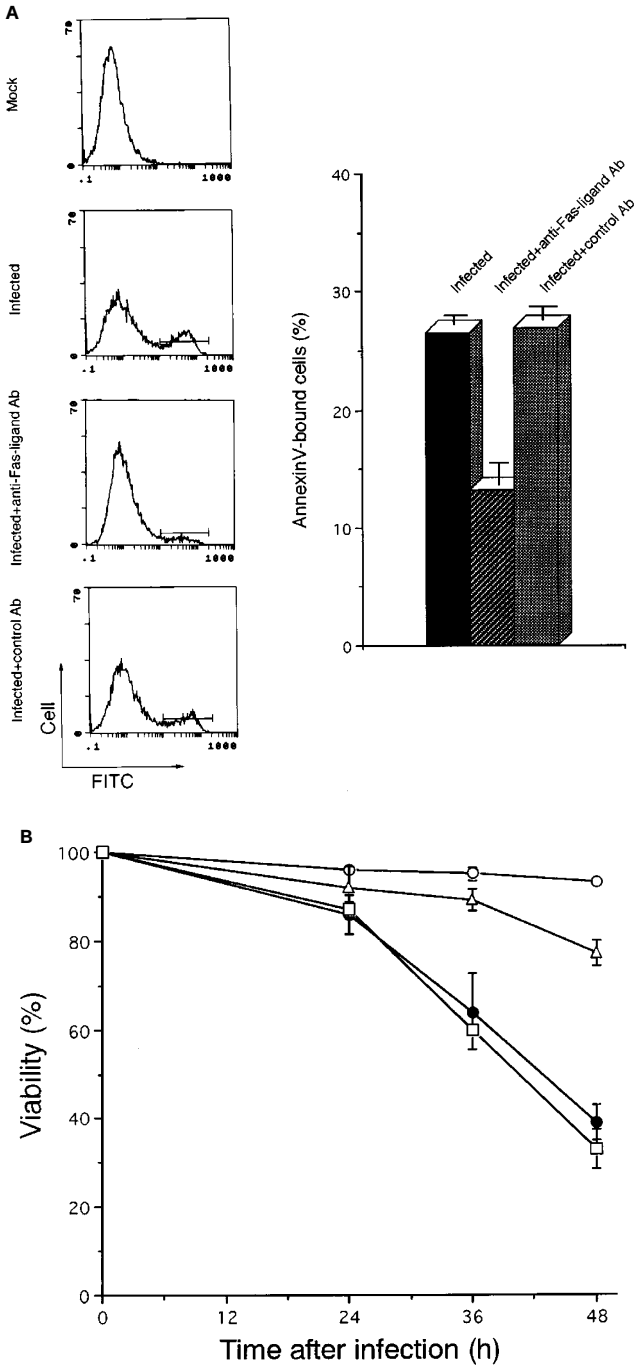


**Figure 6** Inhibition of apoptosis of influenza virus-infected cells by 4H9. HeLa cells were infected with influenza virus in the presence of a saturated amount (10 $\mu$g/ml) of anti-human Fas-ligand monoclonal antibody clone 4H9 or control hamster IgG (Cappel Research Products, USA), and the extent of cell death was determined. (**A**) PS externalization in cells at 12 h after virus infection was determined by flow cytometry. A typical example is shown in the left panels, and the relative number of annexin V-bound cells, shown with horizontal bars, was shown at the right with the mean and standard deviations from three independent experiments. (**B**) Cell viability was determined by a trypan blue exclusion test. The percentages of trypan blue-negative (viable) cells are shown with the mean and standard deviations from three independent experiments. Symbols: (○), mock+no antibody; (●), infected+no antibody; (△), infected+4H9; (□), infected+control IgG

leads to apoptosis of Fas-containing cells, such as antigen-stimulated T-cells (Brunner *et al*, 1995; Dhein *et al*, 1995; Ju *et al*, 1995), anticancer drug-treated leukemia cells (Friesen *et al*, 1996), and human immunodeficiency virus-infected macrophages (Badley *et al*, 1996). Our results indicated that this is the case for influenza virus-infected cells.

Fas-ligand expression seemed to be *de novo* activated upon influenza virus infection whereas pre-existing Fas expression was stimulated. We anticipate that stimulation of Fas expression is needed for apoptosis induction in virus-infected cells since uninfected HeLa cells do not apoptose in the presence of either an agonistic anti-Fas antibody or Fas-ligand-expressing cells unless the cells are manipulated to over-produce Fas (unpublished observations). We have shown that transcription of the Fas-encoding gene is stimulated by a transcription factor called NF-IL6 that is most likely activated by double-stranded RNA-activated protein kinase upon influenza virus infection (Wada *et al*, 1995; Takizawa *et al*, 1996). The results shown in Figure 3 indicated that different mechanisms exist for the regulation of Fas and Fas-ligand expression. Our preliminary experiments showed that an increase in the amount of Fas-ligand upon influenza virus infection also occurs at the transcription level, and transcription regulation of the Fas-ligand-encoding gene is now under investigation.

Externalized PS has been shown to serve as a phagocytosis marker in some apoptotic cells (Savill *et al*, 1993; Zwaal and Schroit, 1997). It is thus of interest whether a change of membrane phospholipid localization in influenza virus-infected cells relates to heterophagic elimination of those cells.

## Materials and Methods

### Cell culture and virus infection

HeLa cells were maintained in Eagle's minimal essential medium (Nissui, Japan) supplemented with 10% fetal bovine serum at 37°C with 5% $CO_2$, and subconfluent monolayers were infected with SP626, a wild-type strain of the influenza A/Udorn/72 (H3N2) virus at a multiplicity of infection of three as described previously (Hatada *et al*, 1992). As a mock-infection control, HeLa cells were similarly treated without the virus.

### Flow cytometric analysis

The virus- or mock-infected cells ($10^6$) were treated with 1% paraformaldehyde for 30 min at room temperature and reacted with anti-human Fas monoclonal antibody clone UB2 (MBL, Japan) (5 $\mu$g/ml) for 30 min on ice. The cells were then supplemented with FITC-conjugated goat anti-mouse IgG (Immunotech, USA) (1 $\mu$g/ml) for 30 min on ice and analyzed in a flow cytometer (EPICS-XL, Coulter). For the analysis of cell surface Fas-ligand, the virus-infected cells were maintained in the presence of KB8301, an inhibitor of matrix metalloproteinase, to prevent proteolytic cleavage of the membrane-bound ligand (Kayagaki *et al*, 1995). The cells were fixed as above and reacted with anti-human Fas-ligand monoclonal antibody clone 4H9 (Tanaka *et al*, 1996) (MBL) (5 $\mu$g/ml) then treated with FITC-conjugated goat anti-hamster IgG (KPL, USA) (10 $\mu$g/ml). To examine the co-expression of Fas and Fas-ligand, the cells were simultaneously treated

with UB2 and 4H9, then with tetramethyl rhodamine isothiocyanate-conjugated goat anti-mouse IgG (Immunotech) (10 $\mu$g/ml) and FITC-conjugated goat anti-hamster IgG and analyzed in a flow cytometer.

### Apoptosis assay

PS exposure to the cell surface was monitored by flow cytometry, making use of fluorescence-labeled annexin V that specifically binds to PS (Koopman *et al*, 1994; Martin *et al*, 1996a). Influenza virus-infected HeLa cells were treated with FITC-labeled annexin V (Bender MedSystems, USA) and PI, and analyzed in a flow cytometer. The cells that were bound by annexin V but only weakly stained with PI, were taken as those with externalized PS. Cells having condensed chromatin were examined under a fluorescence/phase-contrast microscope (BX50, Olympus) after staining with Hoechst 33342. Whether or not the cells had inactive mitochondria was determined by a colorimetric enzyme assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-a diphenyl tetrasolium bromide (MTT) according to the procedure of Mosmann (1983) using a commercial kit (Chemicon International Inc., USA). A $^{51}$Cr release assay was employed to determine an increase of membrane permeability as described previously (Takizawa *et al*, 1993). Briefly, HeLa cells were incubated with $Na_2{}^{51}CrO_4$ (New England Nuclear) for 1 h, then infected with influenza virus. Samples of the medium were taken at the indicated time points and radioactivity was determined. DNA fragmentation assay was done according to the procedure of Tilly and Hsueh (1993). In brief, DNA was extracted from virus-infected cells, labeled with terminal deoxynucleotidyl transferase and [$\alpha$-$^{32}$P]ddATP (Amersham) and separated on a 2% agarose gel then autoradiographed.

### Poly(I)-poly(C) treatment

HeLa cells were maintained in the presence of poly(I)-poly(C) (0.1 mg/ml) (Pharmacia, Sweden) with no serum at 37°C for 24 h. The amounts of cell surface Fas and Fas-ligand were then determined by flow cytometry as described above.

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