Biochemical and Biophysical Research Communications **254,** 5–9 (1999)
Article ID bbrc.1998.9873, available online at http://www.idealibrary.com on IDEAL®

Stimulation Side-Dependent Asymmetrical Secretion of Poly I:Poly C-Induced Interferon-β from Polarized Epithelial Cell Lines

Sachiko Okamoto,* Kiyo Nakanishi,* Yoshihiko Watanabe,† Fumiyoshi Yamashita,* Yoshinobu Takakura,* and Mitsuru Hashida*,[1]

**Department of Drug Delivery Research and †Department of Molecular Microbiology, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan*

Received November 26, 1998

Mode of secretion of poly I:poly C-induced IFN was examined using epithelial cell lines in a bicameral culture system. Although the cell lines formed a tight cell sheet and produced IFN-β following poly I:poly C treatment in spite of its application to the upper or lower compartment, IFN secretion differed between the apical and basolateral cell membranes. When poly I:poly C was applied to the upper compartment, IFN was secreted predominantly from the apical membrane. Inversely, poly I:poly C applied to the lower compartment caused preferential IFN secretion from the basolateral membrane. These results suggest that in epithelial cells poly I:poly C stimulation induces intracellular membrane traffic toward the stimulation side.

Polarized epithelial cells play fundamental roles in the specialized vectoral transport and secretion of proteins in tissues and organs (1–6). Although many studies of polarized epithelial cells have been performed using established cell lines including human colorectal carcinoma Caco-2 (7–10) and Madin-Darby bovine kidney (MDBK) (11) cells, the precise mechanism of the secretion polarity of proteins is not completely understood.

In this context, secretion of cytokines such as interferon (IFN) in polarized epithelial cells have received little attention. The expression of type I IFN gene is usually latent in many cell types and induced by various types of stimulation including viral infection and double-stranded RNA treatment (12–14). Thus, how the secretion of the induced IFN would be controlled in polarized epithelial cells is worthy of study in order to understand the physiological roles of IFN in host defense mechanisms of animal bodies.

In this study, we investigated the mode of secretion of IFN induced by polyriboinosinic acid:polyribocytidylic acid (poly I:poly C) in mouse squamous carcinoma Pam-T (15, 16), Caco-2, and MDBK cells, using the efficient IFN induction method with cationic liposomes (17). Our results show that in all the epithelial cell lines examined poly I:poly C-induced IFN is preferentially secreted from the stimulated membrane domain, providing a novel insight into the regulation of protein secretion.

MATERIALS AND METHODS

Cell lines. Pam-T and MDBK were grown in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml of streptomycin. Caco-2 cells (ATCC HTB 37) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% nonessential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin and used within 37 passages. The medium was changed every 2 to 3 days. Mouse L and human FL cells, used for bioassay of mouse and human IFNs, respectively, were maintained in minimum essential medium (MEM) supplemented with 6 % FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

IFNs and antibodies. Highly purified human fibroblast IFN-β was kindly donated by Toray Co. Ltd. (Tokyo, Japan). Highly purified mouse IFN-β was prepared from NDV-induced L cell IFNs (18). Antibodies used in this work were as follows: sheep anti serum against human IFN-α (19), rabbit anti serum against human IFN-β (20), rat monoclonal antibodies specific for mouse IFN-α (4E-A1) and -β (7F-D3) (21).

IFN induction by poly I:poly C. To examine the optimal conditions for IFN induction, cells (5×10^4 cells/culture) were cultured in 24-well tissue-culture plates for 1 day. After being thoroughly washed with serum-free medium, the cells were treated with various concentrations of poly I:poly C (Pharmacia Biotech, Uppsala, Sweden) mixed with 8 μg/ml lipofectin (Gibco BRL, Gaithersburg, MD) for 2 h. The medium was replaced with growth medium, and the cells were incubated for a further 24 h. The medium was recovered and assayed for IFN activity as described below.

[1] To whom correspondence should be addressed. Fax: 81-75-753-4575. E-mail: hashidam@pharm.kyoto-u.ac.jp.

Abbreviations used: IFN, interferon; poly I:poly C, polyriboinosinic acid:polyribocytidylic acid; MDBK, Madin-Darby bovine kidney.

0006-291X/99 $30.00

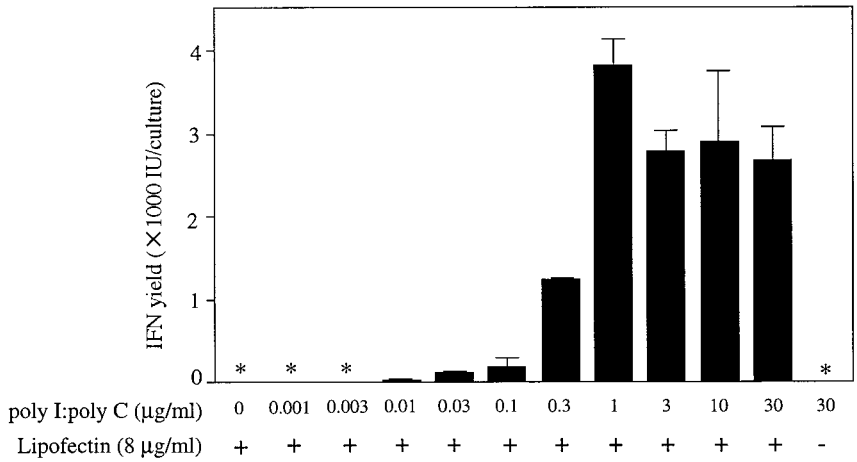

FIG. 1. IFN induction by poly I:poly C–lipofectin in Pam-T cells. Pam-T cells cultured on 24-well tissue culture plates were treated with complexes of various amounts of poly I:poly C and 8 μg/ml lipofectin for 2 h. After removal of the complexes, the cells were incubated in growth medium for a further 24 h, and then the IFN activity in the culture fluids was measured. The values represent the mean ± SE of triplicate determinations. (*) undetectable.

In the experiments investigating the mode of IFN secretion, 1×10^5 cells/well were plated on Transwell filters (Costar, Cambridge, MA; 1 cm^2 culture area) and cultured; generally the cell sheet become impermeable for trypan blue and IFN-β as early as 3 days after cell culture and the impermeable state lasted at least up to day 14 (not shown). On day 5, the cell monolayers were thoroughly washed with serum-free medium, and then treated with poly I:poly C (1 μg/ml)–lipofectin (8 μg/ml) complexes from the upper or lower compartments for 2 h. The medium in both compartments was replaced with growth medium followed by addition of human IFN-β (for Pam-T) or mouse IFN-β (for Caco-2 and MDBK) (finally about 200 IU/ml) to the upper or lower compartments as a permeability marker. After an additional 24 h of incubation, the medium in both compartments was recovered separately and assayed for IFN activity.

IFN assay. IFN activity of the supernatant from cell cultures was measured as described previously (18, 22). In brief, the activity was determined by the reduction in the cytopathic effect of encephalomyocarditis virus (EMCV) on human FL cells for human IFN, and of vesicular stomatitis virus (VSV) on mouse L cells and bovine MDBK cells for mouse and bovine IFNs, respectively. IFN subtypes were analyzed by combinational using of IFN-neutralizing antibodies.

RESULTS

IFN induction by poly I:poly C–lipofectin complexes. To improve the efficiency of IFN induction by poly I:poly C, we utilized a cationic liposome, lipofectin (23), as an additive: suitable complexes of poly I:poly C and lipofectin were good inducers (17). In Pam-T cells, following complexation with lipofectin (8 μg/ml), more than 0.01 μg/ml poly I:poly C induced detectable amounts of IFN and 1 μg/ml poly I:poly C induced the highest titer of IFNs, whereas poly I:poly C alone in-

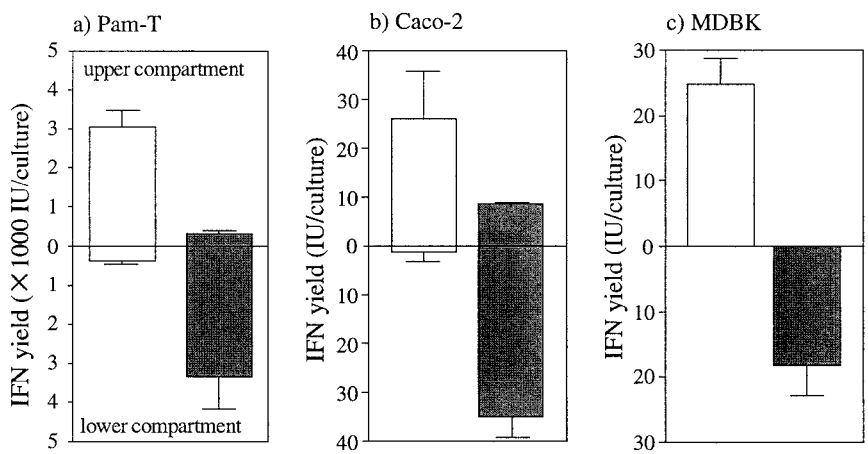

FIG. 2. Polarized secretion of IFN induced by poly I:poly C–lipofectin complexes. Pam-T (a), Caco-2 (b), and MDBK (c) cells were cultured in a bicameral system. Poly I:poly C–lipofectin complexes were applied to the upper compartments (open bar) or lower compartments (shaded bar) and incubated for 2 h. After a further 24 h incubation, the IFN activity in the culture fluids in the upper and lower compartments was individually measured by the appropriate bioassay. The values represent the mean ± SE of triplicate determinations.

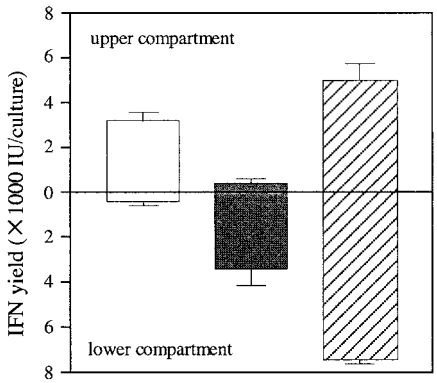

FIG. 3. Mode of IFN secretion of Pam-T cells treated with poly I:poly C–lipofectin complexes. Pam-T cells cultured in a bicameral system were treated with poly I:poly C–lipofectin complexes from the upper compartments (open bar), lower compartments (shaded bar) or both (hatched bar) as in Fig. 2. The cells were cultured further for 24 h, and the IFN activity in the culture fluids in the upper and lower compartments was individually measured as in Fig. 2. The values represent the mean ± SE of triplicate determinations.

duced no detectable IFN even at 30 μg/ml (Fig. 1). Similarly, Caco-2 as well as MDBK cells treated with 1 μg/ml poly I:poly C and 8 μg/ml lipofectin yielded the highest titer of IFNs (data not shown). Thus, complexes of 1 μg/ml poly I:poly C and 8 μg/ml lipofectin were used in the IFN-induction experiments (see below).

IFN production by poly I:poly C was transient; it was detectable at about 3 h after stimulation, peaked at 10–12 h and then gradually declined almost to terminate at 24 h (data not shown). The poly I:poly C-induced IFN consists mainly of type β (>95% of IFN activity) with a little type α (<5%) (data not shown), as neutralized by antibodies, except for MDBK IFN which was not examined for type due to a lack of appropriate antibodies being available.

Secretion polarity of poly I:poly C-induced IFN in a bicameral culture system. The secretion polarity of IFN induced by poly I:poly C was examined using Pam-T, Caco-2, and MDBK cells in a bicameral culture system (Fig. 2). When poly I:poly C–lipofectin complexes were applied to the upper compartments, all the cell lines secreted IFNs mainly into the upper compartments and very little into the lower compartments. On the other hand, when the complexes were applied to the lower compartments, IFN activity was detected predominantly in the lower compartments. This polarized secretion was not affected by simultaneous application of lipofectin alone to the compartment not treated with inducer, indicating that the direction of IFN secretion was due to poly I:poly C-stimulation itself and not to lipofectin treatment (data not shown). In addition, when treated with complexes on both sides, the Pam-T cells secreted comparable amounts of IFN into both compartments (Fig. 3).

In parallel, the impermeability of the monolayer during the secretion polarity experiments was examined using heterologous β-type IFN as a permeability marker. Since IFN-β is highly species-specific, poly I:poly C-induced IFN and marker IFN in one sample can be separately measured by species-specific IFN assay systems, provided the titer of the marker IFN is not too high. Marker IFN applied to the upper compartment was detected entirely in the upper compartment when poly I:poly C treatment was applied to the same compartment. However, it was faintly (1–4% of input) detectable in the lower compartment when the treatment was applied to the lower compartment, and vice versa (Table 1). These "leakages" are not due to cross-reactivity of induced IFNs because they are neutralized by antibodies specific for the added marker IFN, but not by those against poly I:poly C-treated cell-derived IFN (data not shown).

TABLE 1

Marker IFN Permeability through the Cell Monolayer upon Stimulation by Poly I:Poly C-Lipofectin Complexes

Cell line	PolyI:polyC applied to	Marker IFN applied to	% recovery of marker IFN	
			Apical compartment	Basal compartment
Pam-T	Apical	Apical	84 ± 0.4	Nil
	Apical	Basal	**0.9 ± 0.1**	92 ± 0.0
	Basal	Apical	83 ± 1.7	**3.9 ± 1.1**
	Basal	Basal	Nil	100 ± 3.9
Caco-2	Apical	Apical	97 ± 12	Nil
	Apical	Basal	**1.5 ± 0.2**	106 ± 17
	Basal	Apical	99 ± 5.8	**1.6 ± 2.2**
	Basal	Basal	Nil	93. ± 8.7
MDBK	Apical	Apical	98 ±3.2	Nil
	Apical	Basal	**0.5 ± 0.2**	100 ± 0.0
	Basal	Apical	95 ± 5.8	**2.3 ± 2.2**
	Basal	Basal	Nil	101 ± 6.4

DISCUSSION

In this study, we examined the mode of secretion of IFN induced by dsRNA, poly I:poly C, in three kinds of epithelial cells, Pam-T, Caco-2, and MDBK in a bicameral culture system separated by a polycarbonate filter. Under the conditions used in our experiments, all cell types formed a monolayer on the filter which did not allow trypan blue (not shown) or IFN to passively transfer from one compartment to the other, presumably due to the tight junctions. This is the first documentation that mouse epithelial Pam-T forms such a tight monolayer, suggesting that Pam-T will serve as a useful model for studying the polarity of skin epithelial cells in the mouse system.

Although poly I:poly C is a well-known inducer of IFNs, the effective doses of poly I:poly C are so high that they are relatively cytotoxic (24, 25). To overcome this disadvantage, we have established a much more efficient IFN induction method using cationic liposomes including lipofectin (17). Complexes of 1 μg/ml poly I:poly C and lipofectin (8 μg/ml) caused efficient IFN induction in all three types of epithelial cell examined in this work. It is considered that cationic liposomes bind to anionic poly I:poly C to reduce the electronic repulsion of cell surfaces, and thereby the complexes are taken up into the cells more efficiently and then trigger IFN induction.

The poly I:poly C-induced IFN activity was predominantly β-type. Natural IFN-β is commonly N-glycosylated glycoprotein, and its secretion is led by signal peptide (26). Since it has no apparent sequences for targeting intracellular localization or sorting other than the signal sequence for secretion, the secretion is considered to be mediated through default bulk flow of membrane traffics (27). Of interest, however, IFN-induction experiments with Pam-T, Caco-2, and MDBK in the bicameral culture system revealed that IFN secretion occurred mainly from the cell surfaces to which poly I:poly C was applied (Fig. 3), as if epithelial cells recognized the direction of stimulation and secreted IFN predominantly from the stimulation side.

The three types of epithelial cells examined here, differing from each other in animal species and tissue origin, displayed essentially the same results, implying that the stimuli directed asymmetrical secretion of induced IFN is a common phenomenon. Although the underlying mechanisms are unknown, the accompanying data may provide some information about this issue. To make certain of the tightness of cell sheets during the IFN induction experiments, we simultaneously examined the transfer of heterologous IFN-β through the sheets. The marker IFN applied to one compartment was faintly detectable in the opposite compartment only when poly I:poly C stimulation was carried out from the opposite compartment, but not from the same compartment as the marker (Table 1). It is, therefore, unlikely that the transfer of a very small amount of marker IFN was due to destruction of the tight junctions between the cells by poly I:poly C treatment. Rather, it seems that poly I:poly C treatment produced uptake of cytomembrane at that side by pinocytosis and resulted in a 'shortage' of membrane component, consequently increasing transcytosis of the opposite membrane, as a counterbalance, towards the stimulation side, thereby causing predominant secretion of induced IFN and transcytosis of a small fraction of the marker IFN in the opposite compartment towards the stimulated side. When marker IFN was applied to the same compartment as the poly I:poly C treatment, the postulated membrane dynamics from the opposite membrane would not be associated with transcytosis of the marker towards the opposite side. Alternatively, in polarized cells, the routs of signaling of exogenous stimulation might link to the routs of responding processes.

Although further experiments are required to prove these hypotheses, our findings may open up new perspectives on cell polarity, which previously been thought to be rather fixed cell properties and be dependent on the nature of proteins. Anyway, this unequivalent secretion of stimuli-induced IFN, depending on which side is treated by the stimuli, might be meaningful in exerting the physiological effects properly in the situation of host defense against microbes in animal bodies. An apparently similar phenomenon of stimulation-side directed secretion of induced cytokines has been reported for interleukin-8 (IL-8) induced by tumor necrosis factor α and IL-1 on human epithelial cells (28).

ACKNOWLEDGMENTS

This work was supported in a part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan, and the grant of "Basic Research on Drug Innovation" by the Japan Health Sciences Foundation.

REFERENCES

1. Casanova, J. E., Apodaca, G., and Mostov, K. E. (1991) *Cell* **66,** 65–75.
2. Rodriguez-Boulan, E., and Powell, S. K. (1992) *Annu. Rev. Cell Biol.* **8,** 395–427.
3. Pimplikar, S. W., and Simons, K. (1993) *Nature* **362,** 456–458.
4. Matter, K., and Mellman, I. (1994) *Curr. Opin. Cell Biol.* **6,** 545–554.
5. Scheiffele, P., Peranen, J., and Simons, K. (1995) *Nature* **378,** 96–98.
6. Marzolo, M. P., Bull, P., and Gonzalez, A. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 1834–1839.
7. Pinto, M., Robine-Leon, S., Appay, M. D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Siom-Assmann, P., Haffen, K., Fogh, J., and Zweibaum, A. (1983) *Biol. Cell* **47,** 323–330.
8. Matter, K., Stieger, B., Klumperman, J., Ginsel, L., and Hauri, H. P. (1990) *Cell* **60,** 429–437.

9. Hughson, E. J., and Hopkins, C. R. (1990) *J. Cell Biol.* **110,** 337–348.
10. Peterson, M. D., and Mooseker, M. S. (1992) *J. Cell Sci.* **102,** 581–600.
11. Madin, S. H., and Darby, N. B. (1978) *Proc. Soc. Exp. Biol. Med.* **98,** 574–576.
12. Colby, C., and Morgan, M. J. (1971) *Annu. Rev. Microbiol.* **25,** 333–360.
13. Kleinschmidt, W. J. (1972) *Annu. Rev. Biochem.* **41,** 517–542.
14. Kelley, K. A., and Pitha, P. M. (1985) *Virology* **147,** 382–393.
15. Yuspa, S. H., Hawley-Nelson, P., Koehler, B., and Stanley, J. R. (1980) *Cancer Res.* **40,** 4694–4703.
16. Maruguchi, Y., Toda, K. I., Fujii, K., Imamura, S., and Watanabe, Y. (1991) *Cancer Lett.* **60,** 41–49.
17. Okamoto, S., Watanabe, Y., Takakura, Y., and Hashida, M. (1998) *J. Biochem.* **124,** 697–701.
18. Watanabe, Y., and Kawade, Y. (1987) *in* Lymphokines and Interferons: A Practical Approach (Clemens, M. J., Morris, A. G., and Gearing, A. J. H., Eds.), pp. 1–14, IRL, Oxford.
19. Kawade, Y., Watanabe, Y., Yamamoto, Y., Fujisawa, J., Dalton, B. J., and Paucker, K. (1981) *Antiviral Res.* **1,** 167–178.
20. Watanabe, Y. (1983) *Microbiol. Immunol.* **27,** 433–444.
21. Kawade, Y., and Watanabe, Y. (1987) *in* The Biology of the Interferon System 1986 (Cantell, K., and Schellekens, H., Eds.), pp. 197–201, Martinus Nijhoff, Dordrecht.
22. Watanabe, Y., Kuribayashi, K., Miyatake, S., Nishihara, K., Nakayama, E., Taniyama, T., and Sakata, T. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9456–9460.
23. Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., and Danielsen, M. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 7413–7417.
24. Rodgers, R., and Merigan, T. C. (1972) *Crit. Rev. Clin. Lab. Sci.* **3,** 131–162.
25. Carter, W. A., and DeClercq, E. (1974) *Science* **186,** 1172–1178.
26. Weissmann, C., and Weber, H. (1986) *Progr. Nucleic Acid Res. Mol. Biol.* **33,** 251–300.
27. Mostov, K., Apodaka, G., Aroetic, B., and Okamoto, C. (1992) *J. Cell Biol.* **116,** 577–583.
28. Lammers, K. M., Jansen, J., Bijlsma, P. B., Ceska, M., Tytgat, G. N., Laboisse, C. L., and van Deventer, S. J. H. (1994) *Gut* **35,** 338–342.