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Combination Effects of Interferon-γ and Cholera Toxin on Induction of Differentiation of an Insensitive U-937 Clone

Masahide Iwanami, Ken Takeda, Sanju Iwamoto and Kunio Konno

First Department of Biochemistry, School of Medicine, Showa University, 1-5-8, Hatanodai, Shinagawa-ku, Tokyo 142

We examined the combination effect of interferon-γ (IFN-γ) and cholera toxin and the role of cAMP in the induction of differentiation of a differentiation-insensitive U-937 clone, in which the reactivity to differentiation-inducers was decreased. IFN-γ (100 units/ml) or cholera toxin (10^{-9} *M*) alone only marginally induced various differentiation-associated characteristics such as NBT-reducing activity, phagocytic activity, α-naphthyl acetate esterase activity and surface markers. However, when combined with each other, they significantly induced these markers. Other cAMP-inducing agents such as prostaglandin E_2, forskolin, epinephrine and isoproterenol did not induce NBT-reducing activity, either alone or in combination with IFN-γ. However, all these cAMP-inducing agents significantly increased intracellular cAMP levels. Tumor necrosis factor, interleukin 6 or granulocyte/macrophage colony-stimulating factor alone did not induce NBT-reducing activity, but they could induce activity when combined with cholera toxin. These results suggest that enhancement of induction of differentiation by cholera toxin in combination with IFN-γ or other cytokines may not be merely due to increased cAMP levels. There seems to be a transduction signal other than cAMP coupling with cholera toxin to stimulate induction of differentiation of an insensitive U-937 clone.

Key words: Interferon-γ — Cholera toxin — Differentiation — Differentiation-insensitive U-937 clone — Intracellular cAMP level

Human myelogenous leukemia cells can be induced by a variety of chemicals and physiological inducers to differentiate and mature.[1] Interferon-γ (IFN-γ) induces differentiation of a monocytoid leukemia cell line, U-937, and a myeloid leukemia cell line, HL-60, into monocyte/macrophage lineage.[2–5] In addition to its differentiation-inducing activity, IFN-γ has several biological activities including antiviral activity, growth-inhibitory activity and immunomodulatory activity.[6] These biological activities are elicited by binding to the specific cell-surface receptors. However, little is known of the mechanisms by which IFN-γ transmits its various effects from the cell surface into the cell.

We have observed that IFN-γ synergistically induced differentiation of U-937 cells when combined with cAMP-inducing agents such as cholera toxin, prostaglandin E_1 and forskolin (unpublished).

In the present work we isolated a differentiation-insensitive U-937 clone which has relatively low reactivity to various differentiation inducers. IFN-γ induced differentiation only marginally and cholera toxin did not induce differentiation in these cells. However, simultaneous addition of both agents did induce differentiation. Treatment of cells with cholera toxin is known to increase cAMP by activating adenylate cyclase through ADP-ribosylation of the GTP-binding protein N_S.[7–9] Therefore, we examined the role of cAMP in the effects that combinations of IFN-γ and cholera toxin have on the induction of differentiation of an insensitive U-937 clone.

MATERIALS AND METHODS

Reagents RPMI1640 medium was purchased from Grand Island Biological Co., Grand Island, N.Y. and fetal bovine serum (FBS) was obtained from Filtron Pty. Ltd., Victoria, Australia. Nitroblue tetrazolium (NBT) dye, forskolin, cholera toxin, epinephrine, isoproterenol, and α-naphthyl acetate esterase assay kit were purchased from Sigma Chemical Co., St. Louis, Mo. Highly purified natural IFN-α (2×10^7 units/mg protein) and IFN-γ (7×10^7 units/mg protein) were donated by the Green Cross Co., Osaka. Highly purified natural IFN-β (2×10^7 units/mg protein) was donated by Toray Co., Tokyo. Analysis by SDS-polyacrylamide gel electrophoresis indicated that these IFNs were essentially pure. Recombinant human tumor necrosis factor (rHuTNF) was donated by Dainippon Pharmaceutical Co., Osaka. Recombinant human granulocyte/macrophage colony-stimulating factor (rHuGM-CSF) was provided by Sumitomo Pharmaceutical Co., Osaka. Recombinant human interleukin 6 (rHuIL-6) was purchased from Genzyme Co., Boston, Mass. Monoclonal antibody OKM1 (CD11b) was purchased from Sanbio Bv-Biological Products Co., Uden, the Netherlands. FMC17 (CD14) was purchased from Sera-Lab Co., Crawley Down, England. Fluorescein isothiocyanate-conjugated goat IgG $F(ab')_2$ anti-mouse IgG+IgM was purchased from IBL Co., Fujioka. Endotoxin contamination of

these recombinant cytokines was less than 2 ng/mg protein. Prostaglandin E_2 (PGE_2) was donated by Ono Pharmaceutical Co., Osaka.

Cell lines and cell culture The human monocytoid leukemia cell line U-937, established from a patient with histiocytic lymphoma,[10] has been maintained as a suspension culture in RPMI1640 medium supplemented with 10% heat-inactivated FBS. Differentiation-insensitive clones were isolated from U-937 cells by a limiting dilution method. Clone DI-1, which has relatively lower reactivity to various differentiation-inducers than the parent U-937 cells, required more than 100 times the concentration of IFN-γ to induce the same level of NBT-reducing activity as that of the parent U-937 cells. Cells were cultured in a 96-well microplate by adding 0.2 ml of RPMI1640 medium containing 6×10^4 cells, 10% FBS and test materials at the desired concentrations.

Differentiation assay Differentiation was monitored by determining the appearance and accrual of various cellular markers usually associated with maturation of the monocytic elements.

NBT-reducing ability NBT-reducing activity was assayed colorimetrically by the modified method of Baehner and Nathan.[11] Briefly, 6×10^4 cells were suspended in 96-well microplates with 0.2 ml of RPMI1640 medium containing 10% FBS and appropriate test material(s) at the desired concentration(s), and incubated at 37°C. After three days, 0.1% NBT dye and 30 ng of 12-O-tetradecanoylphorbol-13-acetate were added in each well and incubated at 37°C for 60 min in a 5% CO_2 incubator. After incubation, the reaction was terminated by adding 50 μl of 2 *N* HCl to each well of the 96-well microplate and cooling on ice for 30 min. The medium was then discarded, the blue-black formazan deposits were dissolved by adding 0.2 ml of dimethylsulfoxide, and the optical density of the dissolved formazan was measured by spectrophotometry in the 96-well microplates (Immunoreader NJ-2000, Intermed, Tokyo) at 590 nm. The percentage of cells containing blue-black formazan deposits was determined by counting at least 200 cells under a microscope.

Morphological differentiation The appearance of morphological differentiation was assayed on day 5 in stained slide preparations. Slides were prepared by centrifuging in a Cytospin (Shandon Southern Products, Ltd., UK), and stained with May-Gruenwald solution and diluted Giemsa solution. Cells that had increased cytoplasm and an eccentrically placed oblate nucleus with loosely stranded nuclear chromatin were counted as macrophage-like cells. At least 200 cells were examined for each experimental point.

Phagocytic activity Phagocytic activity was determined on day three by the method of baker's yeast digestion.[12] U-937 cells were cultured with test reagents for 3 days. Cells were washed and mixed with 0.2 ml of opsonized yeast particle suspension and incubated for 1 h at 37°C. Cells were then washed again and 0.2 ml of 0.01% fuchsin solution was added to the cell pellet. Viable cells with digested yeast particles were scored as phagocytosis-positive, and at least 200 viable cells were counted in each determination.

Esterase activity Cytochemical staining for α-naphthyl acetate esterase activity was carried out on day 5 as previously described.[13]

Cell surface phenotype expression Binding of monoclonal antibodies (MoAbs) to U-937 cells was determined by direct immunofluorescence and fluorescence microscopy. U-937 cells (6×10^4 cells) were suspended in 0.2 ml of RPMI1640 medium containing 10% FBS and test material(s) at the desired concentration(s). After blocking of Fc receptors with human serum (AB), the cells were incubated with a monoclonal antibody (OKM1 or FMC17) for 30 min in an ice bath, followed by the addition of a fluorescein isothiocyanate (FITC)-coupled goat IgG $F(ab')_2$ antibody directed to mouse IgG+IgM for another 30 min in an ice bath. The cells, suspended in 50% glycerol in PBS, were mounted on glass slides and examined by fluorescence microscopy.

Determination of intracellular cAMP U-937 cells (2×10^6/ml) were incubated with or without the test materials at 37°C for various times. The reaction was stopped by placing the mixture in an ice bath and by addition of 3 ml of cold RPMI1640 medium. After removal of the medium, 0.5 ml of 6% trichloroacetic acid (TCA) was added. Cells were then sonicated for 1 min with a Handy Sonic, model UR-20P, Tomy Seiko Co., Tokyo. TCA-insoluble materials were removed by centrifugation, and TCA in the supernatant was removed by extraction with water-saturated diethyl ether. The samples were lyophilized, and then the content of cAMP was determined with a radioimmunoassay kit (Dupont Co., Boston, Mass.).

RESULTS

Combination effects of IFN-γ and cholera toxin on the appearance of differentiation-associated characteristics in an insensitive U-937 clone After treatment with IFN-γ and/or cholera toxin, the maturation process in the induction of differentiation of U-937 cells was monitored by measuring the appearance of various differentiation-associated characteristics. One of these indicators, reduction of NBT dye, was measured on day 3. IFN-γ (100 units/ml) or cholera toxin (10^{-9} *M*), alone, induced NBT-reducing activity only marginally, but treatment with combined IFN-γ and cholera toxin synergistically induced NBT-reducing activity. Synergistic effects on other cellular markers, such as phagocytosis,

α-naphthyl acetate esterase activity, and morphological maturation were also observed when IFN-γ was combined with cholera toxin (Table I). Combination effects of IFN-γ and cholera toxin were also observed on the cell surface phenotype expression. Treatment of U-937 cells for 3 days with 100 units/ml of IFN-γ or 10^{-9} *M* cholera toxin caused a marginal increase of positive cells for a mature myelomonocyte (OKM1) and a monocyte (FMC17) antibody, while combined use of IFN-γ and cholera toxin resulted in a significant increase in the percentage of cells reacting with both antibodies (Table I).

Dose-dependent effects of cholera toxin and forskolin on induction of NBT-reducing ability of an insensitive U-937 clone in the presence and absence of IFN-γ Cholera toxin alone, at concentrations up to 10^{-8} *M*, could not induce NBT-reducing activity, but in combination with 100 units/ml IFN-γ, cholera toxin dose-dependently induced NBT-reducing activity at concentrations from 10^{-11} *M* to 10^{-8} *M* (Fig. 1A). Forskolin did not significantly induce NBT-reducing activity, either alone or in combination with IFN-γ, up to a concentration of 10^{-5} *M* (Fig. 1B).

Dose-dependent effects of IFN-γ on induction of NBT-reducing activity in the presence and absence of cholera toxin As shown in Fig. 2, IFN-γ alone induced NBT-reducing activity only marginally at concentrations up to

Table I. ombination Effects of IFN-γ and Cholera Toxin on the Appearance of Differentiation-associated Characteristics in a Differentiation-insensitive U-937 Clone

Addition	α-Naphthyl esterase (%)	NBT reduction (%)	Phagocytosis (%)	Morphological maturation (%)	OKM1 (CD11b) (%)	FMC17 (CD14) (%)
None	0.3±0.3	0±0	0.3±0.5	1.2±0.7	0.3±0.4	0.6±0.5
IFN-γ 100 units/ml	1.4±0.4	0.3±0.3	2.1±0.8	4.5±0.9	1.7±0.8	1.6±0.7
Cholera toxin 10^{-9} *M*	1.8±0.8	1.1±1.3	2.7±1.7	2.5±1.8	0.7±0.7	1.0±0.6
IFN-γ 100 units/ml + cholera toxin 10^{-9} *M*	36.0±1.5	23.8±1.6	15.3±6.3	27.9±3.8	28.9±4.1	18.2±3.7

The appearance of various differentiation-associated markers was determined as described in "Materials and Methods." Values are mean±SD of triplicate determinations.

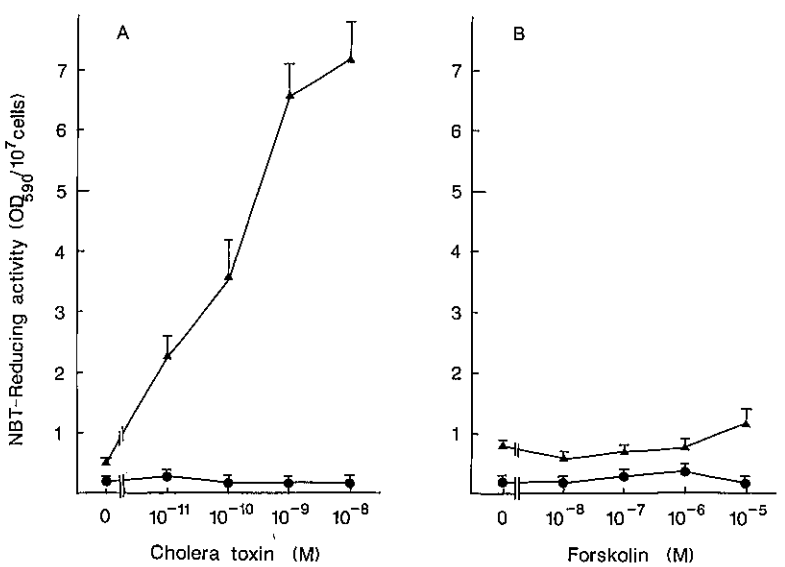

Fig. 1. Dose-dependent effects of cholera toxin (A) and forskolin (B) on induction of NBT-reducing ability of a differentiation-insensitive U-937 clone in the presence or absence of IFN-γ. U-937 cells were cultured for 3 days with the indicated concentrations of cholera toxin or forskolin in the absence (●) or presence (▲) of IFN-γ 100 units/ml. NBT-reducing activity was determined as described in "Materials and Methods." Values are mean ±SD of triplicate determinations.

1,000 units/ml, but when combined with 10^{-9} *M* cholera toxin, IFN-γ induced NBT-reducing activity at concentrations above 10 units/ml. In the presence of 10^{-5} *M* forskolin, IFN-γ did not induce NBT-reducing activity at concentrations up to 1,000 units/ml (data not shown).

Changes in cAMP levels after treatment with cholera toxin or forskolin in a differentiation-insensitive U-937 clone We compared the cAMP levels in the insensitive U-937 cells after treatment with cholera toxin or forskolin. The intracellular cAMP content was measured after exposure of cells to 10^{-9} *M* cholera toxin or 10^{-5} *M* forskolin for various times (Table II). After exposure to cholera toxin, intracellular cAMP levels increased slightly until 30 min, increased remarkably from 30 min to 60 min, and then remained high until 120 min. Although intracellular cAMP levels after treatment with forskolin were lower than those of cells treated with cholera toxin (Table II), they were increased significantly by forskolin in insensitive U-937 cells. IFN-γ did not change the cAMP levels within 120 min (data not shown).

Effects of various cAMP-inducing agents on induction of NBT-reducing activity and on intracellular cAMP levels in combination with IFN-γ in a differentiation-insensitive U-937 clone We further examined the role of cAMP in induction of differentiation of an insensitive U-937 clone using other cAMP-inducing agents such as prostaglandin

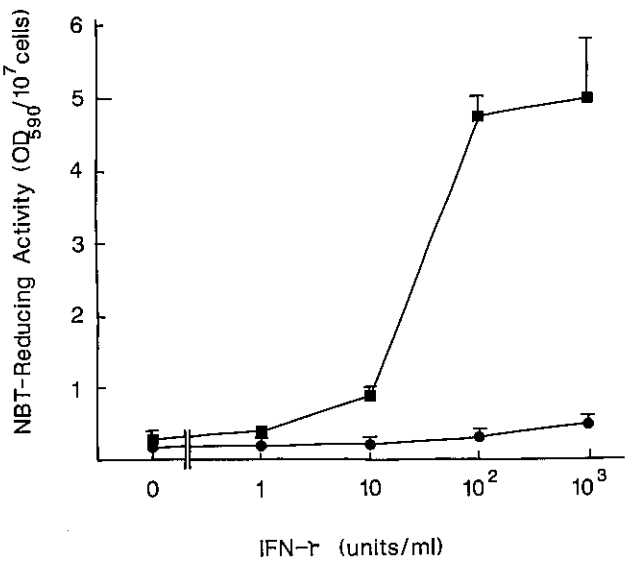

Fig. 2. Dose-dependent effects of IFN-γ on induction of NBT-reducing activity of a differentiation-insensitive U-937 clone in the presence or absence of cholera toxin. U-937 cells were cultured for 3 days with the indicated concentrations of IFN-γ in the absence (●) or presence (■) of 10^{-9} *M* cholera toxin. NBT-reducing activity was determined as described in "Materials and Methods." Values are mean±SD of triplicate determinations.

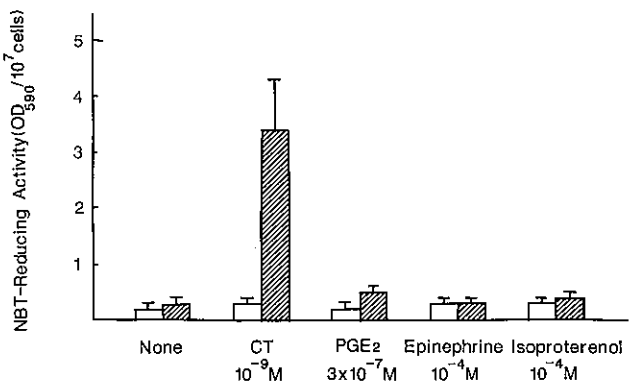

Fig. 3. Effects of various cAMP-inducing agents in combination with IFN-γ on induction of NBT-reducing activity of a differentiation-insensitive U-937 clone. NBT-reducing activity of the insensitive U-937 clone after culture for 3 days with various cAMP-inducing agents was compared in the absence (open bar) or presence (hatched bar) of IFN-γ. Cholera toxin (CT) 10^{-9} *M*; prostaglandin E_2 (PGE_2) 3×10^{-7} *M*; epinephrine 10^{-4} *M*; isoproterenol 10^{-4} *M*. Values are mean ±SD of triplicate determinations.

Table II. Changes in Intracellular cAMP Levels after Treatment with Cholera Toxin or Forskolin in a Differentiation-insensitive U-937 Clone

Test reagent	Cyclic AMP level (pmol/10^6 cells)			
	15 min	30 min	60 min	120 min
None	6.0±1.0	6.3±1.3	10.8±4.4	13.3±0.9
Cholera toxin 10^{-9} *M*	12.3±2.6	21.8±1.0	143.5±7.5	126.1±40.8
Forskolin 10^{-5} *M*	17.0±1.9	15.3±1.9	35.9±9.3	37.6±19.1

Intracellular cAMP was determined as described in "Materials and Methods." Values are mean±SD of triplicate determinations.

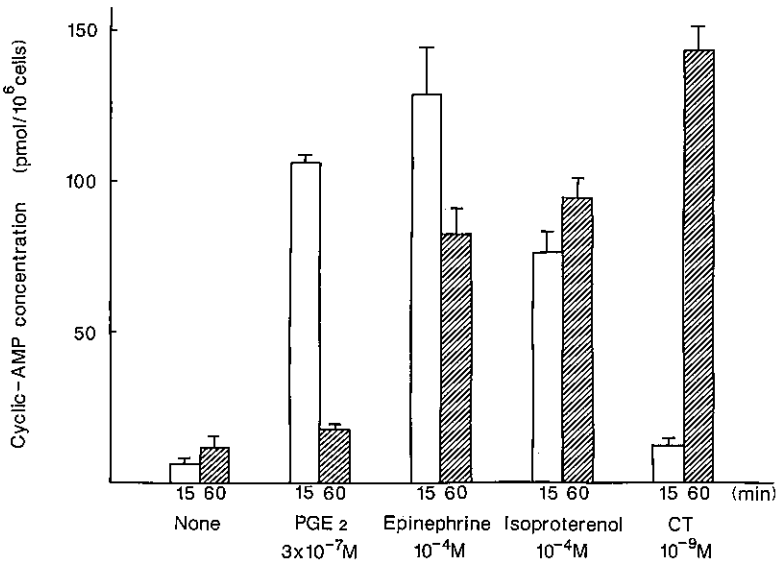

Fig. 4. Effects of various cAMP-inducing agents on intracellular cAMP levels in a differentiation-insensitive U-937 clone. Intracellular cAMP levels after treatment with various cAMP-inducing agents were compared at 15 min (open bar) and at 60 min (hatched bar). Measurement of cAMP was described in "Materials and Methods." Prostaglandin E_2 (PGE_2) 3×10^{-7} *M*; epinephrine 10^{-4} *M*; isoproterenol 10^{-4} *M*; cholera toxin (CT) 10^{-9} *M*. Values are mean±SD of triplicate determinations.

Table III. Effects of Various Cytokines in Combination with Cholera Toxin on Induction of NBT-reducing Activity of a Differentiation-insensitive U-937 Clone

Addition	NBT-reducing activity ($OD_{590}/10^7$ cells)	
	None	Cholera toxin 10^{-9} *M*
None	0.4±0.1	0.4±0.1
IFN-α 1,000 units/ml	0.4±0.1	0.7±0.2
IFN-β 1,000 units/ml	0.5±0.1	0.6±0.2
TNF 1 ng/ml	0.5±0.2	1.5±0.2
IL-6 10 ng/ml	0.5±0.1	2.4±0.3
GM-CSF 10 ng/ml	0.5±0.1	5.0±0.5

NBT-reducing activity was determined as described in "Materials and Methods." Values are mean±SD of triplicate determinations.

E_2 (PGE_2), epinephrine and isoproterenol. As shown in Fig. 3, no cAMP-inducing agent tested alone could induce NBT-reducing activity. In combination with IFN-γ, cholera toxin increased NBT-reducing activity, but no other cAMP-inducing agent did. We examined the intracellular cAMP levels to see if they were increased by these cAMP-inducing agents. As shown in Fig. 4, 3×10^{-7} *M* PGE_2, 10^{-4} *M* epinephrine and 10^{-4} *M* isoproterenol increased cAMP to levels comparable to those obtained with 10^{-9} *M* cholera toxin. PGE_2 increased the intracellular cAMP levels transiently within 15 min, and they declined within 60 min. Epinephrine and isoproterenol also increased the cAMP levels within 15 min, but sustained the increase longer than PGE_2. These results indicate that all cAMP-inducing agents tested could increase intracellular cAMP levels, but that only cholera toxin significantly induced NBT-reducing activity in combination with IFN-γ in insensitive U-937 cells (Fig. 4).

Effects of various cytokines in combination with cholera toxin on induction of NBT-reducing activity of a differentiation-insensitive U-937 clone IFN-α and IFN-β, either alone or in combination with cholera toxin, could not induce NBT-reducing activity of an insensitive U-937 clone. TNF, IL-6 and GM-CSF alone also did not induce NBT-reducing activity, but they could induce the activity in combination with cholera toxin. GM-CSF was as effective as IFN-γ (Table III).

DISCUSSION

IFN-γ dose-dependently induces the differentiation of a human monocytoid leukemia cell line, U-937, into a monocyte/macrophage pathway.[3,4] We previously observed that cAMP-inducing agents such as PGE_1 and isoproterenol enhance the IFN-γ-induced differentiation of U-937 cells. In the present study we isolated a differentiation-insensitive U-937 clone and demonstrated that neither IFN-γ nor cAMP-inducing agents alone could induce differentiation of this U-937 clone. However, when IFN-γ was combined with cholera toxin, it induced differentiation of the insensitive clone. No other cAMP-inducing agent in combination with IFN-γ induced differentiation of insensitive U-937 cells even if it

increased intracellular cAMP to levels comparable to those obtained with cholera toxin. These results suggest that the enhancing effect of cholera toxin plus IFN-γ on induction of differentiation of the insensitive U-937 cells may not be due only to the increase of intracellular cAMP levels. There may be signal pathways other than cAMP/A-kinase for cholera toxin to induce differentiation of U-937 cells.

Forskolin is known to activate adenylate cyclase through a unique mechanism involving direct activation of the enzyme.[14] On the other hand, cholera toxin is believed to increase adenylate cyclase by ADP-ribosylation of a GTP-binding stimulatory protein (N_S).[7-9] Recently, Aksamit *et al.* found that cholera toxin inhibited chemotaxis of a mouse macrophage cell line, and indicated that there was no correlation between cAMP level and inhibition of chemotaxis.[15] Their findings also suggested that cholera toxin can induce certain biological effects via a cAMP-independent process. β-Receptor stimulants are thought to stimulate adenylate cyclase by activating GTP-binding stimulatory protein (N_S).[16,17] Isoproterenol, an α-β receptor stimulant, increased cAMP levels in insensitive U-937 cells, but did not induce differentiation in combination with IFN-γ. Thus, it is possible that cholera toxin stimulates regulators other than N_S.

HA1004, an inhibitor of A-kinase, only marginally inhibited the NBT-reducing activity induced by a combination of IFN-γ and cholera toxin at a concentration of 10 μM, while H-7, an inhibitor of C-kinase, partially inhibited the NBT-reducing activity (approximately 40%) at a concentration of 10 μM in insensitive U-937 cells. H-7 did not inhibit the NBT-reducing activity induced by IFN-γ, but completely inhibited the enhancing effect of OAG (1-oleoyl-2-acetylglycerol), an activator of C-kinase, on IFN-γ-induced NBT-reducing activity in sensitive U-937 cells (data not shown). These observations suggest that C-kinase might be included in the effectors stimulated by cholera toxin in these cells.

Cholera toxin consists of two components, A and B subunits, to which different roles have been assigned.[18] The B subunit binds to gangliosides on the cell surface, and this is required for entry of the A subunit into a cell. The A subunit utilizes intracellular NAD to catalyze an ADP-ribosylation reaction. The B subunit of cholera toxin, which lacks ADP-ribosyltransferase activity and can act as a lectin as well as change the permeability of the cell membrane, did not induce NBT-reducing activity of insensitive U-937 cells in combination with IFN-γ (data not shown).

Olsson and Breitman have shown that PGE_2, cholera toxin or dibutyryl cyclic adenosine 3′:5′-monophosphate, each inactive alone, markedly increased the extent of retinoic acid-induced differentiation of U-937 cells.[19] In previous studies, IFN-γ and cAMP-inducing agents synergistically induced differentiation of sensitive U-937 cells. These results suggest that cAMP/A-kinase may be an important but insufficient signal in induction of differentiation of human myelogenous leukemia cells. cAMP-inducing agents can enhance differentiation induced by various inducers in differentiation-sensitive cells.

Celada and Schreiber showed that protein kinase C activation and mobilization of intracellular Ca^{2+} are essential steps in the IFN-γ-dependent induction of non specific tumoricidal activity in macrophages.[20] However little is known about the signal transduction pathways of IFN-γ in induction of differentiation of myelogenous leukemia cells.

Other differentiation-inducing cytokines such as TNF,[21] IL-6[22,23] and GM-CSF,[23] all inactive alone, induced differentiation of insensitive U-937 cells in combination with cholera toxin, whereas IFN-α and -β did not. It is not clear whether there are any differences between IFN-γ and these differentiation-inducing cytokines in the molecular events that occur after cytokine-receptor interaction and differentiation signals from the cell surface in U-937 cells.

Further studies are necessary to understand the signal transduction in induction of differentiation of myelogenous leukemic cells by IFN-γ and cholera toxin.

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