Human Immune Interferon: Induction in Lymphoid Cells by a Calcium Ionophore

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Human lymphocyte cultures produced interferon after treatment with the calcium ionophore A23187, but not after treatment with potassium ionophore nonactin. Interferon production was detectable between 3 and 6 h after ionophore treatment and reached the maximum level between 12 and 24 h. Ionophore-induced interferon appeared to be immune interferon on the basis of acid lability, lack of neutralization by antibody to leukocyte interferon, slow kinetics of activation of the antiviral state, and molecular characterization by isoelectrofocusing.

Previous studies have shown that production of large amounts of immune interferon may be induced by treatment of human lymphoid cell cultures with the enzyme galactose oxidase (4). This and related findings indicate that oxidation of galactose residues on the lymphocyte membrane may represent at least one specific event which is responsible for lymphocyte activation and induction of immune interferon. It has been reported (8) that ligand activation of surface receptors in a variety of inducible cells is followed by an increase of calcium flow through the membrane and that activation of calcium flux by specific ionophores is often sufficient to stimulate specific cellular responses bypassing the ligand-receptor interaction. For instance, after treatment with calcium ionophores, and in the absence of the specific inducer, endocrine gland cells can produce specific hormones (2, 5, 9, 14), oocytes undergo maturation (16), and lymphocytes may initiate blastogenic transformation (10, 12, 13). As glycoproteins containing galactose residues may be the specific receptors for immune interferon induction, it is also likely that their activation may be mediated by calcium and mimicked by calcium carrier ionophores. This report shows that human immune interferon production may be induced by treatment of lymphoid cell cultures with calcium ionophore A23187 (15) but not by treatment with potassium ionophore nonactin (1), suggesting that divalent cations may act as a "second messenger" in triggering interferon production.

MATERIALS AND METHODS

Lymphoid cell cultures (10^6 cells per ml) were established as previously described by Ficoll-Hypaque gradient sedimentation of peripheral blood from healthy adult donors (4). McCoy medium supplemented with 1% human serum albumin was used as

culture medium. In two instances, lymphocytes from a patient with chronic lymphocytic leukemia or lymphoblastoid cells transformed by Epstein-Barr virus were used.

Calcium ionophore A23187 (Calbiochem, lot 902866) and potassium ionophore nonactin (Boehringer Mannheim Corp., lot 1468304) were added to lymphocyte suspensions (10^6 cells per ml) at concentrations varying from 0.5 μ M to 10 μ M, and the cultures were incubated at 37°C for various times. Some of the cultures were pretreated with actinomycin D (10 μ g/ml, 1 h at 37°C) or with the chelating agent ethylene glycol-bis(β -aminoethyl ether)-*N,N*-tetraacetic acid (1.5 mM) before treatment with the ionophores.

Interferon production was determined after an 18 h treatment of human WISH cell cultures and the activity was measured as previously described by inhibition of Sindbis virus hemagglutinin yield after a single growth cycle (4). The antiviral activity found in the samples was shown to be interferon-mediated in accordance with current criteria (activity against different viruses, inactivity on heterologous cells, blocking by actinomycin D, inactivation by trypsin, lack of inactivation by RNase. Thymidine incorporation was determined at 72 h posttreatment, after 2 h of pulse-labeling with [3 H]thymidine (New England Nuclear Corp. 0.5 μ Ci $\times 10^6$ cells, specific activity 2 Ci/mmol). Viability of the cells (>90%) was verified during the whole incubation period by the trypan blue uptake criterion.

RESULTS

Effect of ionophore treatment on interferon production and DNA synthesis by lymphoid cell cultures from different donors. The results of representative experiments are shown in Table 1 in which interferon production and DNA synthesis have been measured after treatment for 24 h and 72 h, respectively, with 10 μ M of the ionophore.

It may be seen that all the cultures treated with A23187 produced substantial amounts of interferon, whereas none of the cultures treated

TABLE 1. *Effect of ionophore treatment on interferon production and DNA synthesis by lymphoid cell cultures from different donors^a*

Culture	Treatment	Interferon production (NIH reference units per 0.1 ml) ^b	Thymidine incorporation (counts per min per 10 ⁶ cells)
1 ^c	None	<10	72
	A23187	1,000	84
	Nonactin	<10	ND
2 ^c	None	<10	120
	A23187	700	156
	Nonactin	<10	ND
3 ^c	None	<10	94
	A23187	300	91
4 ^c	None	<10	90
	A23187	300	127
5 ^d	None	<10	ND
	A23187	100	ND
6 ^e	None	<10	ND
	A23187	100	ND

^a Abbreviations used: NIH, National Institutes of Health; ND, not determined.

^b We used a 0.1-ml volume in our study; the same titer was obtained with a 1-ml volume.

^c Leukocytes from normal adult donors.

^d Leukocytes from a patient with chronic lymphocytic leukemia.

^e Epstein-Barr Virus transformed B lymphocytes (Daudi cells).

with nonactin showed detectable interferon production. Essentially the same yields were obtained with A23187 at a concentration of 5 μ M, whereas smaller doses induced proportionally lower interferon yields. These doses are similar to, or smaller than, those which have shown biological activity in other systems. Interferon production could, however, be detected in cultures treated with concentrations as low as 0.5 to 1 μ M. With the highest doses of A23187, the inducer could be removed from the cultures by multiple washings at 1 h after its addition, without impairing interferon production. Interferon induction by A23187 was not reduced by pre-treating the inducer with RNase, whereas it was strongly reduced (>90%) or completely abolished when the ionophore was treated at 60°C for 30 min, or when the lymphocyte cultures were treated with actinomycin D or ethylene glycol-bis(β -aminoethyl ether)-*N,N*-tetraacetic acid. The inhibitory effect of actinomycin D suggests that interferon is produced *de novo* after the induction, whereas the suppression by ethylene glycol-bis(β -aminoethyl ether)-*N,N*-tetraacetic acid is consistent with the view that calcium ions play a critical role in interferon induction by A23187. In none of the conditions

studied did the ionophore treatment significantly stimulate DNA synthesis. Lymphocytes remained viable during the whole observation period, as shown by trypan blue exclusion and rate of thymidine incorporation.

Kinetics of interferon production. The kinetics of interferon production were determined in cultures treated with 5 μ M of A23187. Samples were harvested for interferon titration after 1, 3, 6, 11, 24, and 48 h. The results of a representative experiment are shown in Fig. 1. Interferon was detectable as early as 6 h posttreatment; the level increased steadily up to 24 h posttreatment and remained unchanged thereafter. Even though the maximum titer was lower than that induced by galactose oxidase (4), the interferon induced by A23187 and that induced by galactose oxidase were produced with similar kinetics, substantially faster than those observed after induction with conventional mitogens.

Characterization of A23187-induced interferon. The properties of representative samples of A23187-induced interferon, including those produced by chronic lymphocytic leukemia and Daudi cells, were studied in comparison with samples of previously characterized human leukocyte and immune interferons (Table 2). It may be seen that A23187-induced interferon shares with immune interferon all the characteristics tested (acid lability, lack of neutralization by antibody to leukocyte interferon, and slow kinetics of activation of the antiviral state [3]). Molecular characterization of A23187-induced interferon was studied by analytical isoelectrofocusing as previously described (6). Four isoelectric species were found corresponding to pI's of 8.0 to 7.6, 7.0 to 6.46, 5.89 to 5.56, and 5.28 to 4.21. This distribution is similar to that observed with other preparations of immune interferon (6, 7).

FIG. 1. *Kinetics of interferon production in lymphocyte cultures treated with calcium ionophore A23187 (5 μ M).*

TABLE 2. *Characterization of ionophore A23187-induced interferon in comparison with samples of previously identified human leukocyte and immune interferon*^a

Type of interferon	Percent of antiviral activity expressed after		
	1-h treatment at pH 3.0	Preincubation with antibody to human leukocyte interferon	15-min incubation with human WISH cell cultures at 37°C
Leukocyte	100	<10	50
Immune	<10	100	<10
A23187-induced	<10	100	10

^a One hundred reference units of each type of interferon was used. Leukocyte interferon was produced by K. Cantell by Sendai virus induction and was provided through the Antiviral Substances Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Immune interferon was produced as previously described by staphylococcal enterotoxin A induction (11). Antibody to human leukocyte interferon was produced by K. Paucker and provided through the above Antiviral Substances Program. The neutralization test was performed with a serum dilution capable of completely neutralizing 200 reference units of leukocyte interferon. In our assay, the titers reported as reference units correspond exactly to the actual titers observed.

DISCUSSION

Induction of immune interferon by a calcium ionophore, but not by a potassium ionophore, in cultures of normal or transformed lymphoid cells (including Daudi B cells) suggests that divalent cations may act as second messengers in this particular system. Interferon induction occurred in the absence of any detectable increase of DNA synthesis, and this may prove to be an interesting phenomenon because, for the first time, these events appear to be dissociated. The lack of detectable leukocyte interferon in all the preparations tested strongly suggests that ionophore activation of interferon production is specific for immune interferon; if this is the case, calcium flow through the membrane could represent a second specific step, after oxidation of galactose membrane-bound residues, toward genetic derepression of this type of interferon. This view is supported by the finding that galactose oxidase and A23189 show similar kinetics of interferon induction, which are substantially faster than those seen with other mitogens. It is not known yet whether this type of treatment will induce other lymphokines in the absence of detectable blastogenic transformation. This problem is currently under study.

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LITERATURE CITED

1. Asher, J. M., G. D. J. Phillies, and H. E. Stanley. 1974. Nonactin and its alkali complexes—a raman spectroscopic study. *Biochem. Biophys. Res. Commun.* **61**: 1356–1367.
2. Charles, M. A., J. Lawecki, R. Pictet, and G. M. J. Grodski. 1975. Insulin secretion—interrelationship of glucose, cyclic adenosine 3':5' monophosphate, and calcium. *J. Biol. Chem.* **250**:5134–5140.
3. Dianzani, F., L. Salter, W. R. Fleischmann, M. Zucca. 1978. Immune interferon activates cells more slowly than does virus-induced interferon. *Proc. Soc. Exp. Biol. Med.* **159**:94–97.
4. Dianzani, F., T. M. Monahan, A. Scupham, and M. Zucca. 1979. Enzymatic induction of interferon production by galactose oxidase treatment of human lymphoid cells. *Infect. Immun.* **26**:879–882.
5. Garcia, A. G., S. M. Kirpekar, and J. C. Prat. 1975. A calcium ionophore stimulating the secretion of catecholamines from the cat adrenal. *J. Physiol. (London)* **244**:253–262.
6. Georgiades, J. A., M. P. Langford, G. J. Stanton, and H. M. Johnson. 1979. Human immune interferon: isoelectrofocusing analysis. *I.R.C.S. Med. Sci.* **7**:559.
7. Goldstein, L. D., J. A. Georgiades, M. P. Langford, G. J. Stanton, and H. M. Johnson. 1979. Purification and potentiation of human immune interferon activity. *I.R.C.S. Med. Sci.* **7**:560.
8. Gomperts, B. D. 1976. Calcium and cell activation, p. 43–93. *In* P. Cuatrecas and M. F. Greaves (ed.), *Receptors and Recognition, Series A*, vol. 2. Chapman Hall, London.
9. Grenier, G., J. van Sande, D. Glick, and J. E. Dumont. 1974. Effect of ionophore A23187 on thyroid secretion. *FEBS Lett.* **49**:96–99.
10. Hovi, T., A. C. Allison, and S. C. Williams. 1976. Proliferation of human peripheral blood lymphocytes induced by A23187, a streptomycin antibiotic. *Exp. Cell Res.* **97**:92–100.
11. Langford, M. P., J. A. Georgiades, G. J. Stanton, F. Dianzani, and H. M. Johnson. 1979. Large-scale production and physicochemical characterization of human immune interferon. *Infect. Immun.* **26**:36–41.
12. Luckasen, J. F., J. C. White, and J. H. Kersey. 1974. Mitogenic properties of a calcium ionophore A23187. *Proc. Natl. Acad. Sci. U.S.A.* **71**:5088–5090.
13. Maino, V. C., N. M. Green, and M. J. Crumpton. 1974. The role of calcium ions initiating transformation of lymphocytes. *Nature (London)* **251**:324–327.
14. Nordmann, J. J., and P. A. Currell. 1975. The mechanism of calcium ionophore-induced secretion from the rat neurohypophysis. *Nature (London)* **253**:646–647.
15. Reed, P. W., and H. A. Lardy. 1972. A23187: a divalent cation ionophore. *J. Biol. Chem.* **247**:6970–6977.
16. Steinhardt, R. A., and D. Epel. 1974. Activation of sea-urchin eggs by a calcium ionophore. *Proc. Natl. Acad. Sci. U.S.A.* **71**:1915–1919.