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Membrane Events Leading to Interferon-γ Induction by Antigens (41995)

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Abstract. Mitogenic induction of interferon-γ in human peripheral blood mononuclear cells (PBMC) is prevented by enzymatic cleavage of galactose residues on the cell membrane, and by calcium depletion, suggesting that oxidation of galactose on the membrane glycoproteins and activation of a calcium flux across the membrane are critical events for interferon-γ induction in nonspecifically stimulated human PBMC. The same experimental design has been applied to human PBMC cultures enriched of specifically sensitized lymphocytes and stimulated with the respective antigens. The results of these experiments show that also antigenic induction of interferon-γ by purified protein derivative, tetanus toxoid, and MLR requires integrity of galactose residues and calcium intake suggesting that alteration of membrane-bound galactose and activation of a calcium flow are critical triggering events for both specific and nonspecific lymphocyte activation.

Induction of interferon-γ in human peripheral blood mononuclear cells (PBMC) occurs following treatment with the enzyme galactose oxidase or with sodium periodate and it is prevented by the enzymatic splitting of galactose residues on the cell membrane (1). It has also been shown that human interferon-γ can be induced by the calcium ionophore A23187 (2). These findings show that oxidation of membrane-bound galactose residues and/or activation of a calcium flux across the PBMC membrane are sufficient stimuli for the induction of this type of interferon. It has also been shown that various T-cell mitogens fail to induce interferon-γ in human PBMC cultures treated with enzymes to cleave galactose residues or with the chelating agent EGTA to deplete extracellular calcium (1). These data strongly support the view that oxidation of membrane-bound galactose and activation of a calcium flow through the membrane are required steps in the induction of interferon-γ by nonspecific inducers, such as conventional mitogens and oxidizing agents. However these findings do not help define whether interferon-γ induction by antigens in specifically sensitized lymphocytes occurs through the same mechanisms or through other more differentiated events.

To investigate this point we carried out experiments to study interferon (IFN) production by cultures of human PBMC obtained from specifically sensitized donors and treated with the corresponding antigen after cleavage of specific surface components or calcium depletion. The same type of experiment has been carried out also in mixed lymphocyte reactions performed with lymphocytes from several normal adult donors, where IFN production is induced by nonspecific antigenic activation.

Materials and Methods. PBMC cultures were established as previously described by Ficoll–Hypaque gradient sedimentation of peripheral blood (3). Donors were selected according to the various experimental needs as follows: (a) the samples used for stimulation with purified protein derivative (PPD) were obtained from 10 donors showing a strong positive reaction in a routine intradermal tuberculin test; (b) the samples used for stimulation with tetanus toxoid were obtained from 4 adult volunteers vaccinated not more than 3 years before and boosted 10 days before blood collection; (c) mixed lymphocyte reaction (MLR) was performed with PBMC from 10 adult volunteers chosen among those who in preliminary tests showed particularly strong and early reactivity. RPMI 1640 me-

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dium (Flow Laboratories Inc., Irvine, Scotland) supplemented with 5% fetal calf serum, penicillin (200 U/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml) was used as the culture medium. Interferon induction by galactose oxidase (GO; Worthington Biochemical Corp.) or by the calcium ionophore A23187 (Calbiochem) was performed as previously described (2, 3).

Preservative-free PPD and tetanus toxoid (kindly donated by the Research Laboratories of Istituto Sieroterapico Sclavo, Siena, Italy) were used at concentrations of 10 μg and 50 fl/ml/10^7 cells, respectively. MLR was performed according to the conventional assay using a final concentration of 10^6 cells/ml from each of the two donors.

Where indicated, depletion of terminal glycoprotein groups of the PBMC membrane was performed as previously described (1) by treating PBMC suspensions with specific enzymes or enzyme combinations just before addition of the interferon inducers. Briefly, *Vibrio cholerae* neuraminidase (NANase; Schwarz/Mann, 50 U/ml per 10^7 cells, 1 hr at 37°C) was used to cleave *N*-acetylneuraminic acid (NANA) residues and protease-free β-galactosidase (β-Gal; P-L Biochemicals, 800 U/ml per 10^7 cells, 30 min at 24°C) was used to cleave terminal galactose residues. Sequential application of both enzymes was used to split the whole terminal oligosaccharide chain.

After treatment, the cultures were extensively washed, resuspended at the desired concentration in the nutrient medium, and incubated a 37°C for 24 hr (PPD and GO) or 48 hr (tetanus toxoid and MLR). The effect of calcium depletion was studied by performing the induction process in calcium-free medium supplemented with the chelating agent EGTA (1.5 m*M*, pH 7.2).

After each experimental procedure viability of the cultures was established by the dye uptake method and, at the end of the experiments, by the rate of incorporation of [^{35}S]methionine (Amersham, sp act 1050 Ci/mmole, 0.5 μCi/ml, 10^6 cells, 1-hr pulse). Only experiments with cell viability higher than 90% and rate of protein synthesis comparable to control values were considered.

Interferon production was determined after 18 hr of treatment of human WISH cell cultures and activity was measured in international units (IU) as previously described (3) by inhibition of Sindbis virus hemagglutinin yield after a single cycle. Representative samples of interferon were characterized as interferon-γ by conventional tests (instability to acid treatment, inhibition of action by cycloheximide, slow kinetics of activation of antiviral state, neutralization by antibody to interferon-γ, lack of neutralization by antibody to α- and β-interferons).

Cell proliferation was determined by [^{3}H]TdR incorporation as previously described (3).

Results. The data on interferon production by sensitized human PBMC stimulated with PPD, tetanus toxoid, and MLR after cleavage of terminal oligosaccharides on the cell membrane are summarized in Table I. Interferon production by unsensitized PBMC stimulated with GO, used as positive control, has been

TABLE I. INTERFERON-γ INDUCTION BY ANTIGENS IN SENSITIZED HUMAN LYMPHOCYTES TREATED TO MODIFY CELL SURFACE RESIDUES

	Interferon yield[a] after pretreatment with			
Inducer	None	NANase	β-Gal	NANase + β-Gal
None	<4	<4	<4	<4
PPD	106 ± 30	120 ± 15	8 ± 5	<4
Tetanus toxoid	42 ± 15	50 ± 20	4[b]	<4
MLR	160 ± 55	192 ± 64	5 ± 1	<4
GO	1700 ± 48	2048[b]	4[b]	<4

[a] IU/ml ± SD.
[b] No variation is observed in different experiments.

reported. It can be seen that cleavage of NANA residues did not prevent interferon induction by all stimulants tested, while interferon production was substantially prevented by cleavage of exposed galactose residued by β-Gal and completely abolished by cleavage of exposed and unexposed galactose residues by NANase followed by β-Gal.

In no instance was viability of the cells significantly affected by enzymatic treatment, as shown by the unchanged rate of [^{35}S]methionine incorporation (3100 ± 150 cpm in control lymphocytes and 3400 ± 320 cpm in enzyme-treated or untreated stimulated PBMC, as described under Materials and Methods), and by trypan blue uptake (<10%). Furthermore, at the end of the experiment, both control and stimulated PBMC, whether treated or untreated with enzymes, were capable of producing regular and comparable quantities of IFN-α when induced with Herpes simplex virus (an average of 3000 IU/10^6 cells/ml).

As expected (3), in no condition an increase of thymidine uptake was observed when the samples were collected for IFN titration, but an average of eightfold increase of [^{3}H]TdR uptake was found in all stimulated cultures, except those treated with β-Gal or NANase plus β-Gal, when tested 3 days later (not shown). However, the enzymatically treated cells fully recovered the capability of producing IFN-γ when restimulated with the appropriate inducer 4 days after the end of the experiment (not shown).

The results of the experiments where interferon-γ induction was performed under calcium depletion are summarized in Table II. Mean values of at least three experiments have been reported. It can be seen that in every instance interferon-γ production was abolished by calcium depletion.

Characterization of representative samples of IFN induced under the various experimental conditions is reported in Table III. It can be seen that in every instance the IFN produced was IFN-γ with no detectable contamination by the other types of IFN.

Discussion. It has been previously shown that galactose-containing glycoproteins are the sole target structures for oxidative activation of interferon-γ induction (1). The data reported in this paper show that depletion of galactose residues on the cell membrane prevents interferon induction not only in normal lymphocytes stimulated with nonspecific mitogens, but also in specifically sensitized lymphocytes stimulated by their antigens. These findings strongly support the idea that oxidation of galactose residues is a common event in the activation of at least some sets of cells critical for the immune response. In fact activation of IFN induction does not occur in the presence of reducing compounds at sufficiently high concentrations (1).

TABLE II. EFFECT OF CALCIUM DEPLETION IN INTERFERON-γ INDUCTION BY SPECIFIC ANTIGENS AND MITOGENS

Inducer	Interferon production[a] after stimulation	
	EGTA present	EGTA absent
None	<4	<4
A23187	<4	128[b]
GO	<4	2048[b]
PPD	<4	115 ± 25
Tetanus toxoid	<4	32 ± 7
MLR	<4	102 ± 31

[a] IU/ml ± SD.
[b] No variation is observed in different experiments.

That galactose residues are the specific target for oxidative activation of cell proliferation is supported also by the finding that cells treated with β-Gal or with NANase + β-Gal do recover the capability of producing IFN-γ and do increase DNA synthesis when restimulated 3–4 days later, at a time when galactose residues have been restored in the cell membrane (7).

The data do not help define whether the oxidative alteration exerted by antigens occurs directly in the interferon-producing cells or in some other type of precursor cell whose mediation is critical for interferon induction. This hypothesis is currently under study.

Similarly, abolishment of antigenic induction of interferon by calcium depletion supports the view that activation of a calcium flow across the lymphocyte membrane is a second specific step leading to genetic de-

TABLE III. COMPARATIVE PROPERTIES OF IFN PRODUCED IN VARIOUS EXPERIMENTAL CONDITIONS AND IFN-α, -β, -γ

IFN tested	Percentage antiviral activity after							
	2-hr treatment at pH 3	Treatment of cells in the presence of cycloheximide (10 μg/ml)	Treatment of cells at 37°C		Pretreatment with antibody[a] to			
			1 hr	24 hr	IFN-α (A)	IFN-β (B)	IFN-γ (C)	A + B + C
α	100	100	90	100	<10	90	100	<10
β	100	100	100	100	100	<10	100	<10
γ	<10	<10	<10	100	100	100	<10	<10
GO-induced	<10	<10	<10	100	100	100	<10	<10
A23187-induced	<10	<10	<10	100	90	100	<10	<10
PPD-induced	<10	<10	<10	100	100	100	<10	<10
Tetanus toxoid-induced	<10	<10	<10	100	90	100	<10	<10
MLR-induced	<10	<10	<10	100	90	100	<10	<10

[a] Cells were treated for 18 hr either with IFN preexposed in different dilutions (from 100 to 1 reference units) for 1 hr at 37°C to a 10-fold excess of each antiserum or with a mixture of all three. Sheep anti-IFN-α was obtained by Schering Company, Bloomfield, N.J., and had a titer of 200,000 NU/ml in a CPE assay. Sheep anti-IFN-β was obtained by Dr. M. Maeyers, NIAID, NIH, Bethesda, Maryland, and titered 30,000 NU/ml in a CPE assay. Anti-IFN-γ monospecific serum was obtained by Dr. Langford, UTMB, Galveston, Tex., and had a titer of 1.000 NU/ml in a CPE assay.

repression of interferon-γ. Support is found in other inducible systems (5, 6) and in the finding that mitogenic induction of interferon-γ is prevented not only by calcium depletion, but also by the addition of compounds capable of specific blockade of calcium entry or transport (4). This particular finding may be promising not only to provide a deeper insight into the mechanism of activation of the immune responses, such as possible cooperation mechanisms among different sets of cells, but also for some practical implications concerning the possibility of regulating lymphokine-mediated reactions. Some of these studies are being carried out.

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