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Induction of Alpha Interferon by Membrane Interaction between Viral Surface and Peripheral Blood Mononuclear Cells (42041)

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Abstract. Cells infected with viruses and fixed when viral antigens appeared at the cell membrane induced much higher alpha interferon (IFN-α) levels in human peripheral blood mononuclear cells (PBMC) than free virions. Relatively few inducer cells were sufficient for triggering IFN production. Optimal IFN yields depended on inducer/producer cell ratio. The response was peculiar to PBMC as it was not found in other cells in which IFN can normally be induced by free virions. IFN inducing activity was also exerted by live virus-infected PBMC, showing that this type of induction may have physiological importance. These findings confirm that viral induction of IFN-α is activated by some interaction between viral components presented at the cell surface and PBMC membrane. Thus induction of IFN by circulating cells infected by viruses and presenting viral antigens at the surface may be an efficient host defense mechanism. Since IFN yields close to 10^6 international units per milliliter are obtained, this system has potential for large scale production of native IFN-α.

It is widely accepted that viral induction of interferon (IFN) in fibroepithelial cell cultures requires a triggering event which follows virus adsorption, penetration, and uncoating (1, 2). On the other hand, IFN-α can be induced also by nonviral stimuli, such as tumor or foreign cells (3–7), B-lymphocyte mitogens and specific viral antigens (8, 9). It has been demonstrated that viral envelopes, deprived of genetic material, are capable of triggering IFN-α, but not -β production. In fact, virus preparations treated with lipid solvents or uv irradiation, and deprived of nucleocapsid components, completely lose IFN-β-inducing activity, while substantially maintaining the ability to induce IFN-α production (10, 11).

These findings support the idea that virus replication is not necessary for IFN-α induction, and suggest that a membrane interaction is the crucial event leading to its production, although they do not rule out the requirement of internalization of virus component. However, the recent evidence that virus antigen-bearing cells are capable of inducing IFN-α production in human and mouse lymphoid cells (12, 13) confirms the hypothesis that interaction of PBMC with cell membrane bearing viral antigens is a sufficient stimulus and that the engulfment of the inducer is not necessary for the induction process.

The hypothesis that the critical event for IFN-α induction occurs at the cell membrane seems to conflict with the conventional induction of IFN by virus infection. Although these two mechanisms may not exclude each other, one may wonder which is the most prominent.

In this paper IFN yields induced by virus-infected cells fixed with glutaraldehyde, which do not require internalization, have been compared with yields induced by free virions, which may or may not require internalization.

Materials and Methods. *Cell cultures.* Human diploid fibroblasts from skin and muscle, strain E_1SM, mouse L cells, and primary chick embryo fibroblasts (CEF) were grown in Eagle's minimum essential medium (MEM) (Flow Laboratories Inc., Irvine, Scotland), supplemented with 10% fetal calf serum. Human lymphoblastoid Namalva cells were maintained in MEM supplemented with 15% fetal calf serum and 20 m*M* Hepes buffer, pH 7.2. Human amnion WISH cells were grown in RPMI 1640 medium (Flow Laboratories Inc.), supplemented with 10% fetal calf serum.

Preparation of infected, fixed cells. Diploid human embryo fibroblasts from skin and muscle, strain E_1SM, were grown to confluence (2×10^5 cells/cm^2) and then infected with herpes simplex virus type 1 (HSV),

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strain F, at a m.o.i. of 0.1 $TCLD_{50}$ per cell. Twenty-four to thirty hours later HSV-infected and control cells were washed twice with cold PBS and fixed with 0.25% glutaraldehyde or 5% formaldehyde in PBS at +4°C for 10 min. Cells were washed again twice with PBS and once with PBS containing 0.2% bovine serum albumin (BSA) and 0.025% NaN_3. The cells were then scraped with a rubber policeman, resuspended in PBS-BSA-NaN_3 at a concentration of 5×10^5–10^6 cells/ml, and kept at +4°C. Before use, HSV-infected and control cells were extensively washed with PBS. Alternatively, confluent chick embryo fibroblasts (CEF) from 9-day-old embryonated eggs were infected with Newcastle disease virus (NDV) at a m.o.i. of 10 HA/10^6 cells. Twenty-four hours later virus-infected and control cells were fixed and collected as described above. The presence of HSV or NDV antigens on the surface of infected cells was tested by indirect immunofluorescence. The antisera were anti-HSV human serum from a seropositive donor (anti-HSV titer 1:128 in complement fixation test); anti-NDV rabbit serum (titer 1:2000 in neutralization test); goat anti-human (Meloy, Springfield); and mouse anti-rabbit (Behringwerke AG, Marburg Lahn) immunoglobulins, conjugated with fluorescein from commercial sources. This method enabled the detection of envelope as well as internal virus antigens. Cells were fixed when more than 70% showed positive fluorescent staining, i.e., 24–30 hr postinfection. Exploratory experiments had shown that at this time infected cells induce maximal IFN production.

Preparation of infected PBMC. PBMC from healthy donors were infected with HSV at a m.o.i. of 5 $TCDL_{50}$/cell after 3 days of stimulation with 10 μg/ml Con A (Sigma Chemical Co., St. Louis, Mo.) according to previously described procedures (14). After 2 days cells were washed and used to induce interferon in lymphocytes from the same donor. At this time intracellular HSV in the inducer PBMC was $10^{3.5}$ $TCDL_{50}/10^6$ cells, and about 1–5% of the cells were positively stained in the immunofluorescence test.

Interferon induction. In a typical experiment human peripheral mononuclear cells (PBMC), obtained from Ficoll–Hypaque gradient centrifugation of peripheral blood from healthy donors, were incubated with a preestablished number of virus-infected or control cells. The culture medium was RPMI 1640 (Flow Laboratories Inc.) supplemented with 10% fetal calf serum. Supernates were collected at different times and assayed for interferon production content on human WISH amnion cells by Sindbis virus hemagglutinin yield reduction, after a single growth cycle (15). The antiviral activity found was characterized as IFN activity according to current criteria (16), and by neutralization with specific antisera. Neutralization was performed by incubating the IFN in different dilutions (from 100 to 1 international units, IU) for 1 hr at 37°C with a 10-fold excess of the following antisera: sheep anti-IFN-α (Schering Co. Bloomfield, N.J. titer 200,000 neutralization units (NU/ml) in CPE assay); anti-IFN-γ monospecific sera obtained by Dr. Langford, UTMB, Galveston, Texas (titer of 1000 NU/ml in CPE assay). IFN activities were standardized by using the NIH reference standard IFN-α (Ga 23-902-530) supplied by the National Institute of Allergy and Infectious Diseases. The titers are reported in IU.

Viral induction of IFN was performed as previously described (12, 17). Cell viability, determined by the dye exclusion method, was higher than 90% in every experiment. Depletion of adherent cells was obtained by two cycles of adherence incubation in plastic well at 37°C for 1 hr. Blastogenic activity was determined by [^{3}H]thymidine incorporation as previously described (15).

Results. *Induction of interferon by virions or by virus-infected cells in PBMC.* Comparison of the ability of virions and virus-infected cells to induce IFN was made by incubating PBMC with either free HSV virions, HSV-infected cells, or control cells. At preestablished times samples were harvested and brought to pH 2 and assayed for IFN titer. The results of a representative experiment are shown in Fig. 1. It may be seen that IFN yield induced by infected fixed cells was much higher than the yields induced by live virions. On the other hand uninfected, fixed cells do not induce any detectable IFN. The kinetics of IFN release were similar, with the highest IFN production occurring between 12 and 24 hr postinduction, with no further increase thereafter.

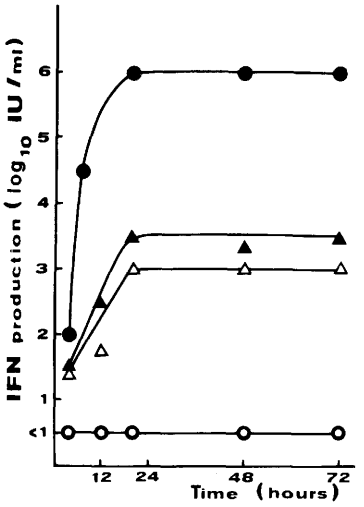

FIG. 1. Kinetics of interferon production by PBMC cultures induced with HSV-infected cells fixed with glutaraldehyde (●), live HSV virions at a m.o.i. of 5 $TCDL_{50}$/cell (▲), live HSV virions at a m.o.i. of 0.5 $TCDL_{50}$/cell (△), and uninfected fibroblasts fixed with glutaraldehyde (○). The ratio between fixed fibroblasts and PBMC was 10^{-2}.

These data suggest that IFN induction by infected, fixed cells occurs through some kind of surface interaction, since fixed cells do not release virions, nor can they penetrate the PBMC. On the other hand the higher IFN yield induced by infected-fixed cells as compared to the yield induced by free virions could be due to a different amount of viral surface antigen or to a different ratio between inducing particles and PBMC. To test this hypothesis, the following experiment was carried out. Control and HSV-infected human diploid fibroblasts or free virions were added at increasing ratios to PBMC cultures. Twenty-four hours later supernates were collected and pH 2 treated for 48 hr before titration. The results of a representative experiment are reported in Fig. 2. Virus-infected cells induced up to 10^6 IU/ml of IFN, while IFN induced by virus alone, did not exceed 10^4 IU/ml. The two IFN curves show a number of differences. Namely, the curve profile of cell-induced IFN is bell shaped since as few as 10 infected-fixed cells (ratio inducer/target 0.00001) induced detectable amounts of IFN; the optimal ratio ranged between 0.01 and 0.001, while a higher ratio is inhibitory. Instead the curve of virus-induced IFN shows that, in order to induce detectable amounts of IFN, free virions require a ratio higher than 0.001 $TCLD_{50}$/cell. The induction is maximal at a ratio of 0.1 and in the present experimental conditions the curve reached a plateau. Similar results were obtained also with cells fixed with formaldehyde (not shown).

IFN induction by uninfected-fixed cells was negligible or undetectable in this system. Also negligible was IFN induction by HSV virions inactivated by glutaraldehyde (Table I).

These findings showed very little variation in repeated experiments (standard deviation ≤ 0.4 $\log_{10}$). In fact, in every experiment infected-fixed cells induced much higher IFN yields than did free virions. No mitogenic activity was shown, as measured by ^{3}H-labeled thymidine incorporation (Table I), while the PBMC response to PHA reached a 100-fold increase of [^{3}H]thymidine incorporation over background levels. Additionally, IFN yield was not affected by removal of glass-adherent cells from the PBMC suspension (Table I), and substantially similar results were obtained using cultures of neonatal mononuclear cells from umbilical cord (Table I). Optimal IFN yields per cell were obtained under conditions of high PBMC density (not shown). Representative samples of IFN obtained by these induction procedures were

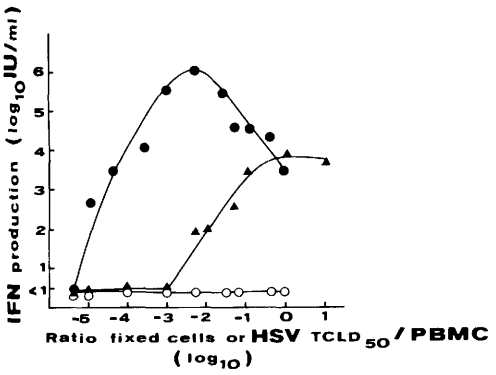

FIG. 2. Interferon induction in PBMC cultures by HSV virions or by HSV-infected cells fixed with glutaraldehyde. PBMC were seeded at a concentration of 10^7/ml and induced with HSV-infected cells fixed with glutaraldehyde (●), live HSV virions (▲), or uninfected fibroblasts fixed with glutaraldehyde (○). Supernates, collected 24 hr later, were titrated as described under Materials and Methods.

TABLE I. IFN PRODUCTION AND [^{3}H]THYMIDINE INCORPORATION BY MONONUCLEAR CELLS AFTER EXPOSURE TO LIVE OR KILLED HSV, UNINFECTED OR HSV-INFECTED CELLS FIXED WITH GLUTARALDEHYDE

Inducer	Stimulated cells	IFN titer (log_{10} IU/ml)	[^{3}H]Thymidine incorporation (cpm × 1000)
None	Mononuclear cells	<1.0	0.39
HSV[a]	Mononuclear cells	2.5[e]	0.42
Glutaraldehyde-treated HSV[a]	Mononuclear cells	1.0	0.42
Uninfected fixed cells[b]	Mononuclear cells	<1.0	0.45
HSV-infected fixed cells[b]	Mononuclear cells	5.9[e]	0.45
HSV-infected fixed cells[b]	Mononuclear nonadherent cells	5.8[e]	N.D.[c]
HSV-infected fixed cells[b]	Mononuclear cells from cord blood	4.7[e]	N.D.[c]
PHA[d]	Mononuclear cells	3.2[f]	32.40

[a] 0.01 $TCLD_{50}$/cell.
[b] 0.01 inducer/producer cell.
[c] Not done.
[d] 10 μg/ml.
[e] Characterized as IFN-α (see Table II).
[f] Characterized as IFN-γ (see Table II).

characterized by neutralization with specific antiserum. Titration before and after acid treatment did not reveal the presence of acid labile IFN, in HSV- or infected cell-induced samples, as shown in Table II.

To define whether this type of response of PBMC may be physiologically meaningful and not limited to cells infected in culture and then fixed we tested also the IFN inducing activity of PBMC infected with HSV and cocultured with uninfected PBMC for the same donor. Specifically, 10^7 PBMC from healthy donors were incubated with 10^6 HSV-infected PBMC in 1 ml of culture medium. Live infected cells have been used in this case, because the amount of live virions present in this culture is not sufficient to trigger IFN production. Because only 1–5% of the IFN inducing PBMC were really infected, as shown by immunofluorescence staining, the effective ratio inducer/producer cells was about 10^{-3}. The results reported in Table III indicate that the kinetics of production and the amounts of IFN produced under these conditions are consistent with

TABLE II. SENSITIVITY TO pH 2 AND NEUTRALIZATION BY SPECIFIC ANTISERA OF IFN INDUCED IN PBMC BY PHA, LIVE HSV, AND HSV-INFECTED CELLS FIXED WITH GLUTARALDEHYDE

IFN tested	Percentage antiviral activity retained after treatment		
	2 hr at pH 2	1 hr at 37°C with antibody	
		IFN-α	IFN-γ
α	100	<10	100
γ	<10	100	<10
PHA induced	<10	80	<10
HSV induced	100	<10	100
HSV-infected cells induced	100	<10	100

TABLE III. IFN PRODUCTION IN CULTURES OF PBMC STIMULATED WITH SYNGENEIC PBMC INFECTED WITH HSV

Inducer	IFN yield (IU/ml)	
	24 hr	72 hr
Expt 1		
Uninfected PBMC	<10	<10
HSV-infected PBMC	3000	2000
Expt 2		
Uninfected PBMC	<10	<10
HSV-infected PBMC	2000	2000

the dose response elicited by fixed, virus-infected fibroblasts reported in Figs. 1 and 2.

Lack of IFN induction by virus-infected cells in cultured cell lines. The extremely effective induction of IFN-α by infected cells supports the view that this type of IFN may be optimally induced by mechanisms which are different from conventional virus induction (11). In fact, if a virus-like stimulus would be involved in this type of induction, it would be equally effective on PBMC and also in other types of cultured cells. To test this assumption conventional virus induction and induction by infected cells were tested in human PBMC cultures and in two established cell lines, Namalva (human lymphoblastoid) and L (mouse epithelioid) cells. For this purpose, a virus which is equally effective as IFN inducer both in lymphoid cells and fibroepithelial cells, NDV, was selected as the inducer. Additionally, this choice would help establish whether this type of induction is peculiar to human fibroblasts infected with HSV, or may occur also with other viruses maturing at the cell surface. The results of Table IV indicate that while NDV was able to induce IFN production, as expected, in all three types of cells, NDV-infected and fixed cells only induced IFN production in normal PBMC cultures and not in any of the cells lines.

Discussion. It was previously shown by us and others (10, 11) that viral envelopes deprived of genetic material were sufficient to induce IFN-α production by lymphoid cells. The findings reported in this paper show that cell-associated viral antigens induce much higher IFN yields than free virus. Relatively few infected cells are sufficient for triggering the response. The decreased inducing activity with a PBMC ratio higher than 0.01 remains unexplained, but it could be due to a less effective interaction between the two types of cells for steric reasons. The response is peculiar to PBMC populations, and is not found in suspended or adherent cell lines which are normally inducible by free virions. Although all the PBMC donors had antibody to HSV, there is no correlation with the immunological status of the donor, since neonatal mononuclear cells produce significant amounts of IFN after induction with virus-infected cells. This finding, and the lack of any blastogenic activity, seem to rule out the involvement of a specific immune response by sensitized cells which is in agreement with results obtained in other systems (13).

TABLE IV. IFN PRODUCTION IN CULTURES OF PBMC AND CELL LINES INDUCED WITH NDV VIRIONS, UNINFECTED, AND NDV-INFECTED CELLS FIXED WITH GLUTARALDEHYDE

Inducer	IFN yield (U/ml)		
	PBMC[a]	Namalva[a]	L[a]
None	<10	<10	<10
NDV[b]	10,000	2000	1600
Uninfected cells[c]	10	<10	<10
NDV-infected fixed cells[c]	80,000	<10	<10

[a] PBMC, Namalva, and L cells were seeded at a concentration of 2×10^6/ml. IFN induction was made with live NDV, NDV-infected, or control chick embryo fibroblasts, fixed with glutaraldehyde. Twenty-four hours later supernates were collected and titrated as under Materials and Methods.

[b] 10 HA/10^6 cells.

[c] 0.1 inducer/producer cell.

All these findings confirm and extend the view postulated by us and by others (5, 10–12) that viral induction of IFN-α in human PBMC cultures is activated by some interaction between viral surface components and PBMC membrane which does not require virus penetration. Furthermore, the high yields of IFN obtained using virus-infected cells in comparison to free virions suggest that the most probable event that leads to the bulk of IFN induction *in vivo* may be an interaction of leukocytes with virus-infected cells rather than with free virions, resulting in an efficient host defense mechanism against the spread of viral infection through circulating cells infected by viruses (18).

Optimal IFN yields per cell are obtained in conditions of high PBMC density, suggesting that lymphocyte interactions play an important role. It is difficult at the present time to formulate hypotheses on the nature of PBMC interactions leading to this sort of amplified IFN production. The participation of macrophages seems to be ruled out since their depletion does not affect final IFN yields; data recently published suggest that NK cells are not required for IFN induction by virus-infected cells, and that the IFN-producing cells express a receptor for com-

plement (13). On the other hand, the lack of response by a lymphoblastoid cell line could reflect the absence of the complex intercellular communications which seem to be required for lymphocyte activation. Experiments are in progress to clarify this point by specific depletion of PBMC populations and reconstitution. This type of approach would also give some insight into the type of cell primarily involved in the production of IFN induced by cells bearing viral antigens. Additionally, deletion of specific components of the PBMC membrane (19) may give some insight into the nature of the triggering event. It is also to be pointed out that the IFN yields reported in this paper are unusually high compared with those obtained by conventional induction procedures, and, therefore, this finding may have practical implications for large-scale production of native IFN-α.

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