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EARLY PLASMA MEMBRANE DEPOLARIZATION BY ALPHA INTERFERON: BIOLOGIC CORRELATION WITH ANTIPROLIFERATIVE SIGNAL[1]

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SUMMARY: Daudi lymphoma cells, of a line sensitive to growth inhibition by alpha interferon, showed dose-dependent plasma membrane depolarization within 10 min after exposure to natural or recombinant alpha interferons (10 to 1000 IU/ml). This biophysical change was detected flow cytometrically by measuring the intensity of fluorescent emission from cells stained with dye indicators of membrane potential. Subclones of Daudi lymphoma cells, resistant to growth inhibition by alpha interferon, showed no membrane depolarization. Parallel results were obtained in initial tests of an isologous pair of T cell and B cell lines which differ in sensitivity to growth inhibition. Thus, decreased membrane potential may herald an interferon signal for antiproliferative action.

Interferons (IFs) comprise a family of pleiotropic bioregulatory proteins (1-4) which commence action shortly after binding to high affinity plasma membrane receptors (5-8). In human cells, selective induction of mRNA transcription can be detected within 10 to 40 min after binding of alpha or beta IFs (9-11); however, early events in signal generation remain problematical (2,12). Rapidly increased cellular levels of diacylglycerol and inositol monophosphate (13) implicate C-kinase, yet no immediate alterations of cytoplasmic free calcium or intracellular pH have been detected (12,13). Other possible signal mechanisms may involve increased rigidity of the plasma membrane (14), increased saturation of phospholipid fatty acids (15), or changes in the cytoskeletal matrix (5,16). That multiple post-receptor

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Abbreviations used: IF(s), Interferon(s); rIFaA, human DNA recombinant alpha interferon (type A); $DiOC_6(3)$, 3,3' dihexyloxacarbocyanine iodide; $DiBaC_4(3)$, bis (1,3 dibutylbarbituric acid) trimethine oxanol; IFa, ultrapure leukocyte interferon alpha; IFg, affinity purified gamma interferon; rIL-2, recombinant interleukin-2, PBS, phosphate buffered saline.

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signaling pathways probably co-exist (1,2,17-19) is indicated by biochemical analyses of IF receptor interactions (6,18) and differing responses of tissue culture subclones with comparable IF binding capacities (8,20,21).

Recently, studies with lymphocytes have demonstrated that several drugs or protein modifiers of biologic responses produce immediate changes of transmembranous ionflux (22-27). Thus we considered that an early shift in membrane potential might correlate directly with IF signaling. For investigation, we choose the monoclonal line of Daudi lymphoma cells (28). This cell line provides a model for the antiproliferative action of alpha IFs (29-32); and its responses to alpha or beta IFs have been extensively characterized (14,17, 30-36). A key consideration was that some available mutant subclones were known to express IF receptors, despite having lost sensitivity to growth inhibition by IFa (30,33,36).

Ionflux changes across the plasma membranes of Daudi cells were analyzed by flow cytometry: the fluorescence intensity of cells stained with specific dyes acting as sensors of the membrane potential was measured (37,38). This non-invasive procedure facilitated sampling of homogeneous cell populations and enabled us to detect a rapid depolarization of the surface membranes of Daudi cells sensitive to growth inhibition by recombinant alpha IF (rIFaA).

MATERIALS AND METHODS

Reagents: The dyes $DiOC_6(3)$ and $DiBaC_4(3)$ were obtained from Molecular Probes Inc., Eugene, OR. Valinomycin and gramicidin were obtained from Calbiochem-Behring Corp., La Jolla, CA. The rIL-2 (4.0×10^6 U/mg) was obtained from Biogen, Cambridge, MA; rIFaA (10^8 U/mg) was a gift from Hoffman La Roche, Nutley, N.J. Affinity purified IFa (2.7×10^8 U/mg) and IFg (6.4×10^6 U/mg) were purchased from Interferon Sciences, New Brunswick, NJ.
Cell Lines and Growth Conditions: The monoclonal line of Daudi cells was derived from a Burkitt's lymphoma (28). Original stocks (Daudi-IF^S), sensitive to the antiproliferative effect of alpha IF (1 U/ml), were supplied by Dr. J.Y. Djeu (see 39) or Dr. A. Kimchi (Rehovoth, Israel). Mutant subclones (Daudi-IF^r), resistant to the anti-proliferative action of alpha IF (up to 500 U/ml) and selected in the laboratories of Dr. Kimchi (33) and Dr. I. Kerr (17,30), were supplied by Dr. Kimchi or Dr. B. Williams (see 39). Daudi-IF^r stocks were maintained with IFa (100 U/ml), but the resistant phenotype was stable for at least 10 population doublings without IFa. An isologous pair of lymphoblastoid cell lines, CCRF-SB (B-cell) and H-SB2 (T-cell), derived from the peripheral blood of a boy with T-cell leukemia (40), were supplied by Dr. J. Minowada (Buffalo, NY) (see 39).

Cells were grown as suspensions in plastic or spinner flasks. The culture medium was RPMI 1640 with L-glutamine, 25 mM Hepes buffer (GIBCO, Grand Island, N. Y.), penicillin-streptomycin and 10% NuSerum (Collaborative Research, Cambridge, MA). A single lot of the latter formulation of growth factors and filtered serum proteins was used for all experiments. Cells were maintained in continuous logarithmic growth (20-24 hr doubling time) at densities between 2 and 10×10^5/ml. This condition proved critical for comparison between experiments (41). Daudi cells vary considerably in growth rate depending upon the frequency of media changes, with a prolonged lag phase in all fresh medium (31). In our protocol half of the medium was exchanged every 24 hr, after being totally replaced 72 hr prior to each experiment.
Flow cytometric determination of membrane potential using $DiOC_6(3)$: The method has been described (42,43): An EPICS V flow cytometer (Coulter

Electronics, Hialeah, FL) was used for data acquisition and analysis. Each cell suspension contained 10^6 cells/ml in complete medium. The $DiOC_6(3)$ dye was added in DMSO (1% v/v, maximum) to a concentration of 25 nM. This was not toxic during experiments: fluorescence intensity maintained a steady-state level indicating no change in membrane potential (no decreases in cell viability). The correlation of fluorescence intensity with the membrane potential was tested by changing extracellular potassium concentrations ($[K^+]$): Increasing $[K^+]$ from 6 mM up to 130 mM proportionately decreased the fluorescence intensity, reaching a minimum 130 mM (At 130 mM, the extracellular and intracellular $[K^+]$ equalizes in lymphocytes). It was also tested by ionophore molecules with known effects (37): gramicidin, 10 ug/ml, considerably decreased while valinomycin, 1 ug/ml, increased the membrane potential. That $DiOC_6(3)$ measured only cytoplasmic and not mitochondrial membrane potential changes was tested by using 30 mM sodium azide (43): after 5 min incubation, fluorescence intensity changes resembled those without mitochondrial enzyme decoupling.

Flow cytometric determination of membrane potential using $DiBaC_4(3)$ dye: The relevance of the fluorescence intensities as measured with $DiOC_6(3)$ dye was doubly checked by applying $DiBaC_4(3)$ (38). Flow cytometric analysis was performed as above and by Wilson et. al. (43). Cells were washed with PBS to remove extraneous protein, treated with biologic test reagents then stained with the dye (150 nM). In contrast to $DiOC_6(3)$ which is a positively charged dye, $DiBaC_4(3)$ is a negatively charged dye. Thus, a fluorescence intensity decrease signifies hyperpolarization. Fluorescence intensity of the $DiBaC_4(3)$-stained cells is stable for 10 min only, so that histograms were taken 2 min after staining. The sample tubing was equilibrated with a 150 nM solution of $DiBaC_4(3)$ before a histogram was taken.

RESULTS

Effect of rIFa on the fluorescence intensity of $DiOC_6(3)$ stained Daudi cells: Cells in complete medium were equilibrated with $DiOC_6(3)$, then treated with varied concentrations of rIFaA. The rIFaA, from 10 U/ml to 1000 U/ml, decreased the fluorescence intensity $DiOC_6(3)$-stained Daudi-IF^S cells (i.e. depolarized) in a dose dependent manner (Fig 1A). Untreated controls showed no decrease. Gramicidin (10 ug/ml) decreased the fluorescence intensity of the $DiOC_6(3)$-stained Daudi-IF^S cells, even beyond that measured with 1000 U/ml of rIFaA (Fig 1C). Typical bit maps, ungated and gated, are shown in Fig 1B and 1C. No change was observed in the light scatter with the 1000 U/ml rIFaA treatment, indicating stable cell geometry. Neither of the Daudi-IF^r subclones exhibited decreased fluorescence intensity when treated with up to 1000 U/ml of rIFaA (not shown). These had been grown without IFa for several doublings (39).

Comparisons of fluorescence intensity changes of $DiOC_6(3)$-and $DiBaC_4(3)$-stained Daudi-IF^S cells treated with rIFa, IFaA, IFg or rIL-2: Aliquots of Daudi-IF^S cells pretreated with 100 U/ml rIFaA for 16 hr and untreated control cells were stained with 25 nM $DiOC_6(3)$. Fluorescence histograms are compared in Fig 2A: rIFaA pretreated cells exhibited lower fluorescence intensities than controls and results with IFa were similar. As expected, gramicidin pretreatment (5 ug/ml) lowered and valinomycin pretreatment (1 ug/ml) increased the fluorescence intensities of control cells indicating depolarization and hyperpolarization respectively (Fig. 2A). Other aliquots

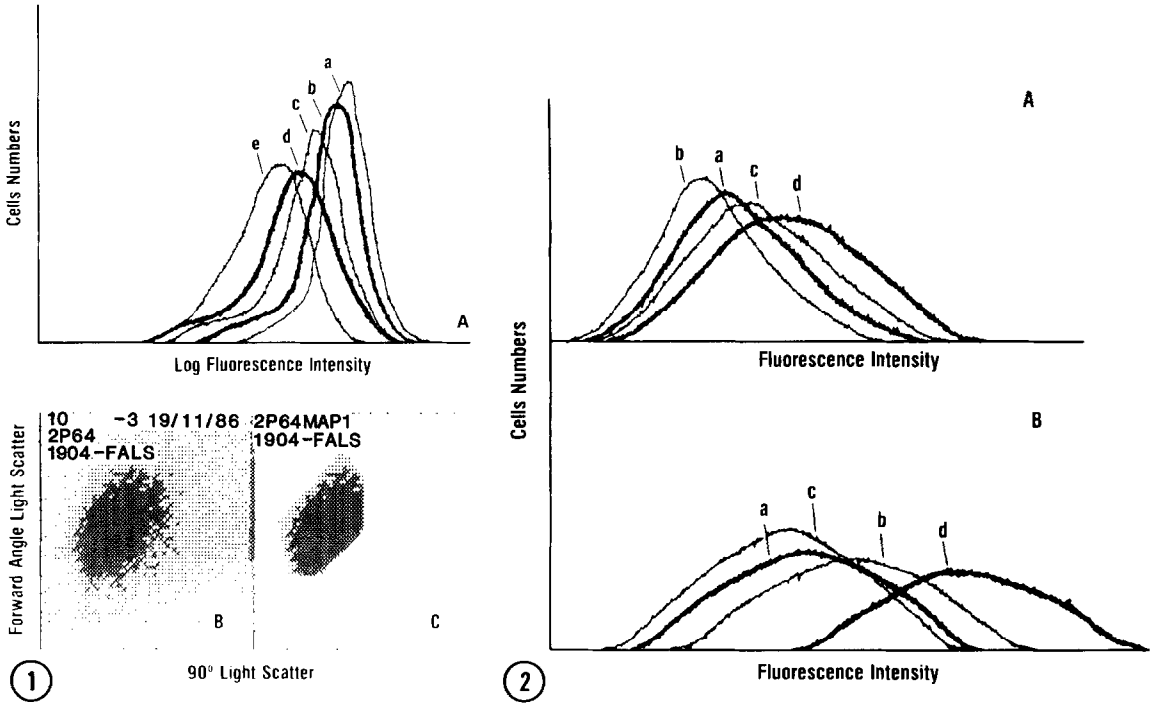

Fig. 1. A. Log fluorescence intensity of 25 nM $DiOC_6(3)$ stained Daudi-IF^S cells: a) no treatment; b) 10 min after 10 U/ml rIFaA; c) 10 min after 100 U/ml rIFaA; d) 10 min after 1000 U/ml rIFaA and e) 10 min after 5 ug/ml of gramicidin. B. Typical bit map of Daudi cells obtained under experimental conditions described in the Materials and Methods; C. The same conditions as B, but after applying electronic gating.

Fig. 2. Histograms comparing the fluorescence intensity changes of A. $DiOC_6(3)$-stained and B. $DiBaC_4$-stained Daudi-IF^S cells (log fluorescence) A: a) control;b) 100 U/ml rIFaA at 16 h, c) 1 ug/ml valinomycin and d) 100 U/ml rIL-2; B): a, b, and c are the same treatments as with A and d) 5 ug/ml gramicidin.

of the same rIFaA-treated and control Daudi-IF^S cells were washed once with PBS, resuspended in PBS, and stained with 150 nM $DiBaC_4(3)$. Fluorescence histograms of $DiBaC_4(3)$-stained control cells, rIFa pretreated cells, and cells treated with gramicidin 5 ug/ml were compared. The higher fluorescence intensities of rIFaA or gramicidin pretreated cells (Fig. 2B) indicated depolarization (due to the negatively charged dye). Valinomycin pretreated cells showed lower fluorescence intensities indicating hyperpolarization (Fig. 2B). Daudi-IF^r cells showed no membrane potential changes: tests with or without rIFaA pretreatment (100 U/ml), using either the $DiOC_6(3)$ or the $DiBaC_4(3)$ dyes, produced superimposable histograms (not shown). These cells nevertheless did change fluorescence intensities with gramicidin or valinomycin treatment (not shown).

Treatment of $DiOC_6(3)$-stained Daudi-IF^S cells with rIL-2 (100 U/ml) resulted in a fluorescence intensity increase within 6 to 8 min, a result opposite to that obtained with rIFaA (Fig 2A). IFg 1000 U/ml produced no fluorescence intensity change.

Effect of rIFaA with paired B-cell and T-cell lines: Aliquots of cells from the isologous CCRF-SB and H-SB2 lymphoblastoid lines were stained with $DiOC_6(3)$ and exposed to rIFaA (100 U/ml). Experimental conditions matched those above in the tests of Daudi cells. In repeated challenges with rIFaA, CCRF-SB cells maintained a baseline fluorescence intensity at times up to 60 min. H-SB2 cells showed a decrease of fluorescence intensity indicative of depolarization, although the effect was slower (30 min) than in Daudi-IF^S cells (10 min). Previous comparisons with IFa had shown that growth of the CCRF-SB line was not inhibited (up to 500 U/ml for 48 hr), whereas growth of the H-SB2 line was moderately inhibited (39).

DISCUSSION: Our experiments demonstrated that rIFaA treatment of Daudi-IFs cells produces plasma membrane depolarization within 10 min, while Daudi-IF^r cells always failed to respond. The phenomena were consistent using dye indicators of membrane potential with either negative or positive charge. Based upon previous studies in lymphocytes, we can assume that a dose-related membrane depolarization initiates signal generation (22-27,43). Preliminary data (Aszalos and Grimley, unpublished) indicates that change in the ionfluxes of potassium is a critical factor. Absence of depolarization in two Daudi-IF^r subclones which do respond to common effects of IFa on suppression of exogenous TdR incorporation (32), induction of oligoadenylate synthetase (30), and accumulation of cytomembranes (39), suggests linkage of rapidly induced ionflux in Daudi-IF^S cells to an anti-proliferative signal. Preliminary comparisons of H-SB2 and CCRF-SB cells supported this conclusion.

The highly sensitive technique of flow cytofluorometric analysis proved advantageous. Some previous analyses of biophysical or biochemical parameters of IF action (6,12,45) could not be quantitated directly with intact cells or were not sensitive during the 10 to 40 min time frame of gene activation defined by early mRNA induction (9-11). Along with the membrane depolarization presently observed at 10 min, only the phenomena of an immediate increase of whole cell diacylglycerol (13), and an increased rigidity of the plasma membrane evidenced by electron spin resonance measurements at 30 min (14) appear to approximate initial events in signal generation by human alpha IF. Enhanced iodide uptake and a change in the membrane potential of rat thyroid cells after exposure to mouse IF also was briefly noted (44).

Signal generation by IF is transient (46), so that continuous receptor occupancy is required for prolonged mRNA transcription (9,46-48). Depolarization of Daudi-IF^S cells for up to 16 hr after continuous treatment with 100 IU/ml of rIFaA was of interest, since receptor internalization and down-regulation after 2 to 5 hr is well recognized (7,49). It suggests that a

fraction of activated receptors may be sufficient for membrane depolarization and a persistent anti-proliferative signal.

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