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DYNAMIC ANALYSES OF LYMPHOBLAST MEMBRANES EXPOSED TO ALPHA INTERFERON USING FLOW CYTOMETRY AND FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING

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Abstract

Interferons represent a major group of the biologic response modifiers which exert multipotent effects upon cell growth, cytodifferentiation and immune functions. Previous experimental studies with alpha interferon (IFN-α) have suggested that modulation of transmembrane signaling could be a critical determinant in the bioregulatory diversity. To determine whether any initial changes at the plasma membrane would directly correlate with one or more actions of IFN-α, we investigated cultures of Daudi lymphoblasts which are uniquely susceptible to growth inhibition. Complementary biophysical techniques were applied. In one approach, changes in plasma membrane ion flux were measured by flow cytometry, using a fluorescent dye indicator of membrane potential: Cells briefly exposed (5–10 min) to a DNA-recombinant IFN-α2 (100 to 800 U/ml) manifested a consistent plasma membrane hyperpolarization (-60 to -90 mV) which could be blocked by ouabain. In a second approach, changes in diffusion coefficients of plasma membrane-associated macromolecules were determined by measuring the fluorescence redistribution after pulse photobleaching (FRAP): Individual plasma membrane proteins (sIgM, Leu 12 or Leu 16) were labelled with FITC conjugated goat antibodies [F(ab')$_2$ or Fab'] or with phycoerythrin-B conjugated monoclonal mouse antibodies. Statistical comparisons of cells exposed to IFN-α2 for 10 to 30 min showed immediate 27 to 88% increases in mean lateral diffusion rates. Mutant Daudi cells, cloned for resistance to growth inhibition showed no plasma membrane hyperpolarization with IFN-α2 (up to 1000 U/ml), and baseline lateral diffusion coefficients matched those of IFN-α2-treated, non-resistant cells. We conclude that biophysical status and responses of the plasma membrane must be closely linked to the molecular mechanisms of anti-proliferative signal transduction.

Key Words: Membrane potential, ion flux, lateral diffusion, membrane fluidity, interferon, lymphoblasts, Daudi cells, Burkitt's lymphoma.

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Introduction

The binding of a biologic regulatory molecule to its receptor on the plasma membrane initiates a series of transmembranous molecular changes which generate the signal(s) leading to biological action (18,74). Alpha interferon (IFN-α) proteins are multipotent cytokines (52,65,66,79) which bind to common subsets of cell surface receptors (11,23,59). The specificity or intensity of signal generation by an IFN-α may be determined, to some extent, by receptor binding affinities (42,58,71); however, it is now widely hypothesized that the pleiotropic actions of a single IFN-α type are best explained by multiple signal transduction pathways (14,24,52). Transmembrane events after receptor binding therefore have become a critical focus for investigation (48,54,76). Some recent studies have implicated cytoskeletal elements (54) or inositol lipid metabolism (76) in the IFN-α signaling mechanism.

Biophysical analytic approaches afford the advantage of detecting early plasma membrane changes in intact, viable cells. Using cells from the continuous monoclonal line of Daudi lymphoblasts (38), which are exceptionally susceptible to growth inhibition by IFN-α (64), we previously were able to demonstrate an early change in plasma membrane ion flux by measuring the redistribution of fluorescent dyes sensitive to membrane potential (28). Measurement of fluorescence redistribution after photobleaching (FRAP) is another sensitive biophysical method and was developed as a means to investigate lateral diffusion either of membrane-associated protein macromolecules tagged with a chromophore (including receptors) or chromogenic lipid analogs (3,20, 32,33,45). Typically, cells have been incubated with a fluorescein-coupled antibody which can be excited by photon pulses in the visible wave range. After thorough washing to remove any excess label, a spot on the plasma membrane is subjected to a microlaser pulse of sufficient energy to quench the fluorophores without cross-linking proteins (75) or disrupting cell integrity. Recovery of fluorescence in the bleached spot depends upon redistribution (lateral diffusion) of labelled molecules from neighboring portions of the cell surface. We have employed an instrument system in which redistribution is sequentially measured (for up to 10 min) by an attenuated laser beam in a scanning mode, and lateral diffusion is calculated from equations developed for a spherical surface (40). Such measurements by FRAP can provide data which is complementary to studies of plasma membrane ion flux in transmembrane signaling (20,34,67) and we now report some early changes in the dynamics of Daudi cell plasma membranes following brief IFN-α treatments.

Materials and Methods

Cell Cultures

Monoclonal Daudi cells were derived from a Burkitt's B-cell lymphoma (38). A spontaneous mutant subclone (herein designated Daudi-IFr) resists growth inhibition (21,37). These cells were cultured as suspensions in plastic flasks using RPMI 1640 with 25 μM HEPES buffer (Gibco, Grand Island, NY) and 10% NuSerum (Collaborative Research, Cambridge, MA). Log-log growth conditions (doubling time of 24–28 h) were consistently maintained (28), and uniformity of the cell cycle distribution was monitored by propidium iodide staining and flow cytometry (25,28). Previous studies have shown that cell cycle consistency is essential for reproducible expression of IFN-α receptors (72) and for comparison of biophysical parameters between different experiments (5,6,70).

Biochemicals and Fluorescent Reagents

Alpha-2 recombinant IFN-α (IFN-α2) was obtained from Schering Corp., Bloomington, NJ and ultrapure leukocyte interferon was purchased from Interferon Sciences, New Brunswick, NJ. Aliquots were prepared from lyophilized stock by dilution into complete medium described above and frozen at −70°C. Activity is given in anti-viral units (U) and was periodically verified with thawed samples as previously described (57). Ouabain was purchased from Sigma Chemical Co., St. Louis, MO. The negatively charged oxonol dye, bis, 1,3 dibutylbarbituric acid, trimethine oxonol [DiBaC4(3)] was obtained from Molecular Probes Inc., Eugene, OR. Concanavalin A (Con A) was obtained from Sigma Chemical Co., St. Louis, MO and made up to 0.5 mg/ml in stock solutions; aliquots were frozen for long term storage at −70°C. Goat $F(ab')_2$, μ-chain specific anti-human IgM (0.98 mg protein/ml) conjugated with FITC (absorbance ratio 1.1) was purchased from Tago (Burlingame, CA). Monovalent Fab' fragments were prepared from the latter by reduction with 2-mercaptoethanol, followed by alkylation with iodoacetamide and extensive dialysis against PBS at 4°C (7). Highly purified mouse monoclonal antibodies to the human B cell antigens, Leu-12 and Leu-16, were obtained as standardized (25 μg immunoglobulin/ml) phycoerythrin-B (PE-B) conjugates from Becton Dickinson Monoclonals, Burlingame, CA.

Labelling of Cells with Flourescent Antibodies

Aliquots of 10^6 cells were sedimented at 300 xg for 7 min in 5 ml plastic tubes, transferred to 1.5 ml microtubes and washed x1 in ice cold endotoxin-free Hanks BSS (Sigma Chemical Co., St. Louis, MO) with 3mM sodium azide to inhibit endocytosis (16). After washing, cells were resuspended in 50 μl of the same buffer, then incubated at 4°C after addition of labelled antibody (20 μl) for 30 min (anti-Leu 12 or anti-Leu 16) or 60 min (anti-IgM fragments). Following labelling, cells were washed as above x2 and resuspended into 100 μl of the same buffer. Each of the antibodies produced a strong ring fluorescence. In some experiments, 20 μl of Con A (100 μg/ml) was introduced for 5 to 10 min after the labelling was completed.

Membrane Potential Measurements

Membrane potential was measured indirectly with the negatively charged oxonol dye, DiBaC4(3), which redistributes at the cell surface in response to altered ion fluxes. The methods used were previously detailed (1,28,73): cells were sedimented, washed (x1) with ice cold phosphate buffered saline (PBS), resuspended in ice cold PBS at a density of ca 25×10^6 cells/ml and maintained in an ice bath. For experimental testing, aliquots of 10^6 cells were prepared in a total volume of 1 ml of PBS and equilibrated for 5 min at ambient temperature in a sampling vial. IFN-α2 (100 to 1000 U/ml) was then introduced and the sample was incubated at ambient temperature for 6 min. In some experiments, cells were pre-treated with ouabain for 1 min prior to addition of IFN-α2. Lastly, the oxonol dye was added and equilibrated for 2 min. The fluorescent intensity of cells thus tested remains stable for up to 10 min (see 73). IFN-α2 effects on intensity of fluorescence emission were quantitated by flow cytometric analysis in a Becton-Dickinson FACScan System, utilizing a 15mV argon ion laser tuned to 488 nm for fluorescence excitation and "C 30" software for signal processing (Becton Dickinson, Mountain View, CA).

Interferon treatments for FRAP

For tests of immediate IFN-α2 effects, antibody-labelled cells were further incubated in buffer with IFN-α2 (800 U/10^6 cells, total volume 100 μl), and equilibrated at ambient temperature. For tests of delayed effects, cultures at a density of 5×10^5 cells/ml were incubated with IFN-α2 (100 U/ml) at 37°C for up to 28 h, then labelled with antibody as previously described.

FRAP Techniques

Work was performed at an ACAS 470 work station (Meridian Instruments Inc., Okemos, MI). This is a computer controlled scanning laser instrument (inverted microscope) with image analysis programs (high speed data acquisition interface with 80286 based computer). Samples were mounted in capillary microchambers fashioned from conventional glass slides and #1 coverslips separated by Scotch double-coated tape (#665, 3M Co., St. Paul, MN). Microchambers measured approximately $3 \times 22 \times 0.1$ mm (width × length × depth) and accommodated up to 5 μl of cell suspension which was introduced with a micropipette. Chambers were charged immediately before use and were sealed with silicone at both edges to prevent desiccation and cell flow during analysis. Under these conditions, cells labelled with fluorescent antibodies or treated with the other reagents remained >95% viable for up to 4 h when maintained at 4°C or ambient temperature. Nevertheless, fresh aliquots were aliquoted from the original preparations after every 30 to 60 min to ensure freshness. Chambers were inverted on the computer controlled specimen stage for epi-illumination, with the #1 coverslip contacting an ×63 Zeiss Neofluor objective (N.A.1.25) using low fluorescence immersion oil (Type B, Cargille Laboratories, Cedar Grove, NJ).

Fluorescence was excited with a 5W argon ion laser (Innova 90–5, Coherent Inc., Palo Alto, CA) tuned to 488 nm. Laser power was set at 400mW, and additional attenuation was provided by a 90% neutral density filter. The zero order laser beam was blocked by an optical light stop and a collimated first order laser beam of adjustable intensity was reflected into the epi-illumination path of an inverted microscope using a standard dichroic FITC filter cube (485/22 BP excitation filter, 510 DLP dichroic mirror, and 515 LP barrier filter). With the oil immersion objective, Gaussian beam diameter was <1 μm. Computer control of the laser beam through an acousto-optic modulator (IntraAction Model 80A) provided for either a low intensity "scan strength" to locate cells or to monitor fluorescence redistribution, or a high intensity "blast strength" to photobleach. Intensity values at each X-Y stage coordinate of a given raster scan were measured with a photomultiplier tube (Hamamatsu model 50-12) and

transformed into 2-D pseudo-color images or 1-D profiles of fluorescence emissions. Prior to each set of analyses, a 2-D low intensity field scan was used to locate immobilized cells which exhibited a uniform rim of fluorescent labelling. Spot photobleaching was performed at the waist and edge of selected cells. A 1-D scan line and the photobleaching position were set with a moveable cursor. FRAP was measured by a pre-selected series of intermittent low energy 1-D scans (5–10 second intervals) including the full cell profile and the bleached zone.

Redistribution rates and lateral diffusion coefficients were computed according to a normal mode analysis for round surfaces developed by Koppel (40). The ACAS 470 program evaluates both mobile and immobile components. Essentially, both the lateral diffusion constant (D) of mobile fluorophores, and the fraction of fluorophores which is mobile (recovery fraction) on the experimental time scale are derived from a mathematical integration of increments in fluorescence ratios, comparing intensity changes both at the bleached edge and the unbleached surfaces. The normal mode analysis defines a quantity called μ, which represents the calculated center of fluorescence for each normalized post-bleach scan. The immobile component causes μ to decay to a constant offset from zero, and this offset is found by averaging the μ values for the last 10% of post-bleach scans. The computer program subtracts the offset from the μ value for each recovery time point and plots the logarithm. Thus, the time scale must be sufficient to allow completion of the recovery. A linear best fit is found, and its slope represents the rate of decay of the mobile component of μ from which (D) is calculated. The ACAS program estimates the log of the mobile component of μ at time zero and calculates the ratio of the mobile component to the total μ at time zero. This is taken to be the mobile (recovery) fraction (R).

FRAP measurements were performed at ambient temperatures, and data for each experimental category was averaged from repeated tests (from two to six times) with separate samples of cells. Up to 44 measurements were obtained in each category and statistical comparisons were performed by means of the student's two tailed t-test. Measurements were not conducted on cells with inappropriate label distribution, and rare cells showing (R) of $<10\%$ or values of (D) with $<50\%$ confidence intervals were excluded.

Results

Membrane Potential Change Induced by IFN-α2 and Effect of Ouabain

The measurable membrane potential span of Daudi cells was established by methods described previously (1,28), using scaled concentrations of extracellular [K+] (5–140 mM). As previously noted with the recombinant alpha-A subtype of interferon (28), IFN-α2 also produced an early shift in membrane potential of Daudi cells in present experiments. This effect was dose related: plasma membrane hyperpolarization became progressively stronger as IFN-α2 concentrations were increased from 100 to 800 U/ml (Fig. 1). Ouabain, a potent inhibitor of $[Na^+]/[K^+]$ ATPase (30,35), produced membrane depolarization of Daudi cells, when it was used at a concentration of 30–40 μM in preliminary tests. At a lower concentration (15 μM), ouabain produced no membrane depolarization, but protected the cells against IFN-α2 hyperpolarization (Fig. 1). Full protection occured only in the range of 100–400 U/ml of IFN-α2.

Analyses of Antibody Labelled Daudi Cells by FRAP

In preliminary work with the ACAS 470, a variety of instrumental settings were tested using Daudi cells labelled with FITC-conjugated anti-IgM or with PE-B-labelled anti-Leu 12. Linear scans disclosed two peaks indicating that the fluorescent antibody was associated predominantly with the plasma membrane, while mean background fluorescence was usually far $<5\%$ (Fig. 2). Optimal recoveries of fluorescence (usually $>60\%$) were obtained following a photobleaching pulse (0.16 mJ) which reduced the fluorescence intensity from 40–70% of its initial level (Figs. 2, 3). Low intensity scanning energy was calibrated at approximately 1–5% of the photobleach intensity and was optimized prior to each set of measurements by performing a series of prescans for up to twice the expected duration of the experiment. Using a pilot specimen the scan beam strength was adjusted to a level which caused well under 5% photobleaching. Under these conditions, μ graph recoveries of Daudi cells were reproducibly asymptotic (carried to completion), between 400 to 500 sec (Figs. 4, 5). Post-recovery integrity of the cells was shown by repeated bleachings and dye viability tests.

The nature of the FRAP technique requires use of antibody probes, such as anti-sIgM, which may of themselves introduce changes in the membrane fluid dynamics (49,56). For comparative studies, we therefore analyzed 3 different plasma membrane antigens (Table 1): sIgM, Leu-12 and Leu-16. Antibodies specific for IgM μ-chain included both the $F(ab')_2$ and Fab' fragments. Individual diffusion coefficients (D) for each of the antigens monitored (Table 2) were within a range of 5.3 to 7.7 $\times$ 10^{-10} cm^2/sec, with mean recovery fractions (R) in a range of 45–80% (Table 2). Both ranges matched previous FRAP data relating to lymphocyte surface proteins (5,16,19,31,44). There was no obvious correlation of (D) with several fold differences in antigen molecular weights, antibody fragment size or either of the two different fluorophore conjugates (Table 1). In accord with some recent data (5); however, the mean diffusion rate of the Daudi cell sIgM labelled with FITC-Fab' fragments was 33% higher ($p<0.01$) than the mean diffusion rate of sIgM reacted with $F(ab')_2$.

The work with PE-B as a fluorophore for FRAP was new, but proved advantageous: the fluorescent yield at 488 nm is several fold higher than for an equivalent fluorescein label (51,69) and the PE-B molecules are more stable than fluorescein during photon bombardment (69). Preliminary direct comparison of FITC-labelled anti-Leu-12 and PE-B-labelled anti-Leu 12 showed similar lateral mobilities of the Leu 12 antigen, and results with an Fab' PE-B anti-kappa chain were similar to the results with Fab' FITC-anti-μ chain (Balint E, Grimley PM, unpublished data).

Analyses of IFN-α2-Treated Daudi Cells by FRAP

FITC-antibody or PE-B-antibody labelled Daudi cells were incubated with IFN-α2 or with polyvalent unlabelled Con A as a "treatment control". With all test antigens, treatment with IFN-α2, for periods of up to 30 min, significantly increased the mean (D), with a range of 7.9 to 11$\times$$10^{-10}$ cm^2/sec (Table 2). In preliminary tests of Daudi cells pretreated with IFN-α2 for 28 h, (D) for sIgM was also increased (not shown). In contrast, Con A consistently decreased the mean (D), with a range from 4.0 to 6.1 $\times$ $10^{-10}$$cm^2$/sec. Previous data indicated that Con A should decrease lateral mobility of plasma membrane proteins (29,47,78). Changes in mobile fractions (R) sometimes

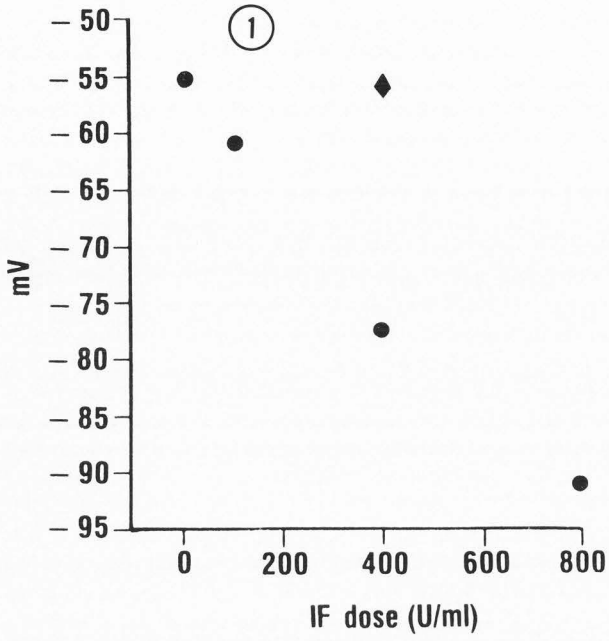

Fig. 1. Shifts of Daudi cell membrane potential calculated in mV (−55 to −90 mV), related to increasing concentrations of IFN-α2 (U/ml). Point marked as solid diamond (at −55mV) shows protective effect of 15μm ouabain when added before IFN-α2 (400 U/ml).

Table 1. Molecular weights of antibodies, fluorophores and Daudi cell antigens tested by FRAP

Antibody	Fluorophore	Antigen	Molecular Weight
Fab′			47,000
$F(ab')_2$			114,000
IgG			145,000
	Fluorescein		384
	Phycoerythrin-B		240,000
		sIgM	900,000
		Leu 12	95,000
		Leu 16	35,000

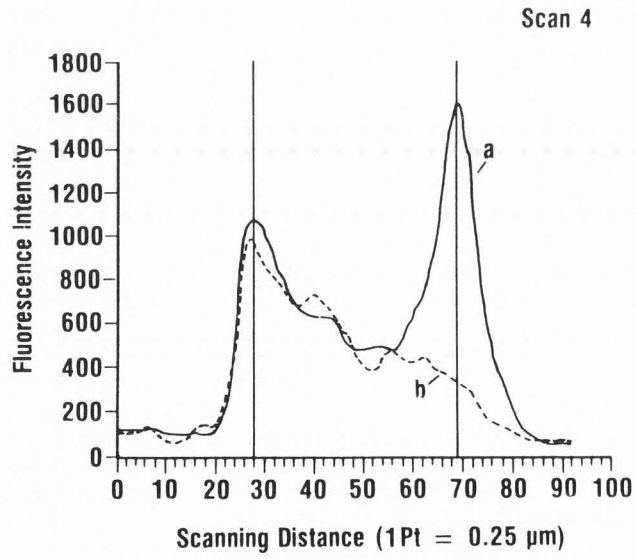

Fig. 2. Low intensity fluorescent scans of a Daudi lymphoblast labelled with FITC-conjugated anti-IgM Fab′ showing fluorescence intensity (arbitrary units) vs. distance scanned by the laser (points at 0.25 μm intervals). a) Average of three linear scans (Scans 1–3) before the photobleaching pulse. Edge peaks and waist diameter of the cells (ca 15 μm) are typical. b) First scan (Scan 4), 10 sec after photobleaching (67%), note stable flourescence intensity at unbleached rim of cell.

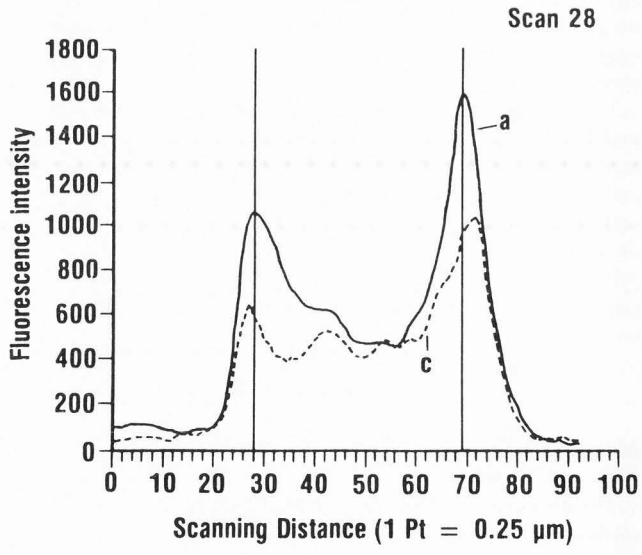

Fig. 3. Scan 28 (c) at 464 sec after photobleaching (the identical cell and same units as in Fig. 2). The essentially completed recovery fraction (R) was 96% (calculation explained in Materials and Methods). A decreased fluorescence intensity of the opposite edge of the cell is probably due to combined effects of dye redistribution and minimal bleaching (<5%) by the repetitious post-bleach scans. The normal mode analysis for round cells (40) compares both cell edges and thus compensates for these changes both in the computations of (R) and the lateral mobility (D).

Table 2. Results of FRAP measurements on Daudi-IFs cells

Antibody	Fluorophore	Treatment	D^c ($\times 10^{10}$cm^2/sec)	N^d	P^e	R^f (%)
α-IgM	FITC[a]	Control	5.8 ± 2.3	44		60 ± 23
F(ab′)$_2$		IFN-α2	11 ± 2.8	29	<0.001	80 ± 20
		Con A	4.4 ± 1.4	17	<0.02	80 ± 20
α-IgM	FITC	Control	7.7 ± 2.1	29		66 ± 20
Fab′		IFN-α2	9.8 ± 2.8	20	<0.01	67 ± 27
		Con A	6.1 ± 1.8	17	<0.05	32 ± 21
α-Leu 12	PE[b]	Control	5.4 ± 2.2	26		61 ± 25
		IFN-α2	7.8 ± 2.8	31	<0.001	78 ± 12
		Con A	4.0 ± 1.4	14	<0.02	52 ± 25
α-Leu 16	PE	Control	5.3 ± 1.2	36		54 ± 24
		IFN-α2	7.9 ± 1.4	21	<0.001	62 ± 27
		Con A	4.1 ± 1.1	14	<0.10	74 ± 30

[a]Fluorescein isothiocyanate
[b]Phycoerythrin-B
[c]Diffusion coefficient (mean ± SD)
[d]Number of measurements
[e]Probability in student two-tailed t-test, compared to control values
[f]Mobile fraction

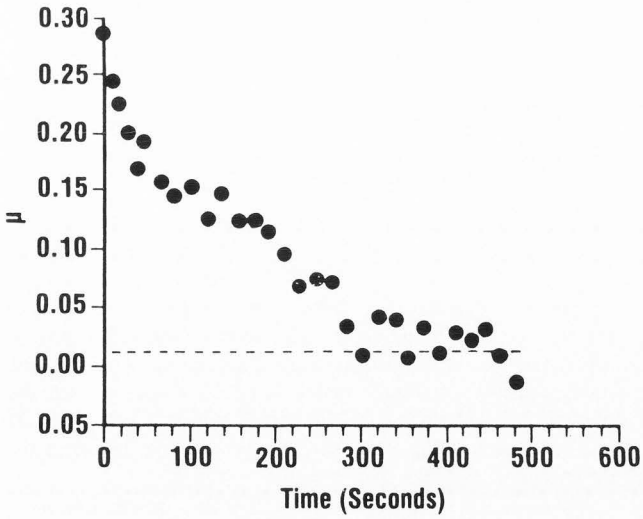

Fig. 4. ACAS 470 plot of μ vs. time (seconds), after photobleaching of the identical cell scanned in Fig. 2, according to the normal-mode analysis of spherical cells (40) in which μ represents the center of fluorescence (see Materials and Methods).

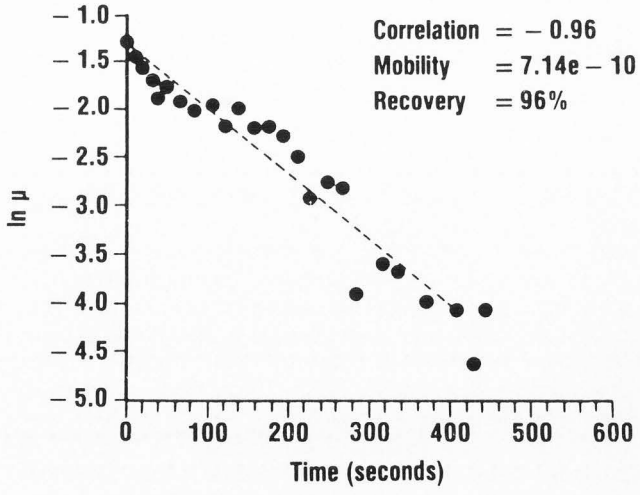

Fig. 5. ACAS 470 regression plot of ln μ vs. time (seconds), after correction for the μ offset (analysis of same cell as Figs. 2–4, see Materials and Methods). The computation for (D) was 7.14×10^{-10}cm^2/sec (correlation 0.96).

paralleled the changes in D and in two instances data were statistically significant: a) there was an increased (R) after incubation of FITC-anti-IgM $F(ab')_2$-labelled cells with IFN-α2 ($p < 0.02$), and b) there was a decreased (R) after incubation of FITC-anti-IgM $F(ab')_2$ or PE-B-anti-Leu 12 labelled cells with Con A ($p < 0.02$).

Comparative Analyses of Daudi-IFr Cells

In view of the above findings, it became of interest to compare FRAP results with a mutant subclone of Daudi cells (Daudi-IFr) known to resist the antiproliferative action of alpha interferons and the concomitant suppression of **c-myc** oncogene activity (37). We have found that Daudi-IFr cells behave similarly to other alpha interferon resistant Daudi clones (17,26,64,72) by remaining sensitive to induction of 2'-5' adenylate synthetase, and to inhibition of thymidine kinase (Grimley PM, Walsh D, Rupp B, unpublished data). Previously, we had shown that Daudi-IFr cells resisted the membrane potential changes induced by type A alpha interferon (28). The latter was reconfirmed, using Daudi-IFr cells which were continuously maintained in 1000 U/ml of ultrapure leukocyte interferon or treated once a week with 100 U/ml. Under either of these maintenance conditions, plasma membranes of the Daudi-IFr cells did not hyperpolarize when incubated with 100 U/ml of DNA-recombinant IFN-α2. They only partially lost resistance after growth for two weeks (>8 mass doublings) in medium without interferon, and simultaneously became susceptible to hyperpolarization (Aszalos A, Grimley PM, unpublished data).

In FRAP experiments, preliminary data with PE-B-anti-Leu 16 labelled cells indicated that (D) values in Daudi-IFr cells were very close to the values previously observed in their parental Daudi-IFs cells **after** IFN-α2 treatment. The mean (D) value for control Daudi-IFr cells in log-log growth was $9.4 \pm 1.0 \times 10^{-10}$ cm^2/sec (11 measurements) and after IFN-α2 was essentially unchanged ($8.7 \pm 0.8 \times 10^{-10}$ cm^2/sec). The significance of these (D) values compared to (D) values for Daudi-IFs cells was $p < 0.001$ (see Table 1). These higher (D) values in PE-B-anti-Leu 16 labelled Daudi-IFr cells were observed for up to one week after removal of leukocyte interferon from the maintenance growth media, during which time there had been at least four mass doublings.

Discussion

Effects of IFN-α2 on the cell genome and biosynthetic pathways are very rapid. New messages begin to appear within 10 min and their transcription may peak at 1 to 2 h (41). Thus, the experimental window for exploring primary effects of IFN-α2 at the receptor and post-receptor levels are narrow. Non-disruptive biophysical measurements may offer the best opportunity to observe rapid plasma membrane changes which approximate primary signaling events. The methods of membrane potential measurement and FRAP which we have tested obviously require several minutes of cell preparation, but can be promptly deployed. Evidence that alpha interferon signals are short-lived (4), and that alpha interferon, attached to its receptors, cumulatively stimulates cells (4,61,68), lends further credence to these measurements. Other studies (8,10) including electron spin resonance (53), also have suggested changes in the plasma membrane environment after alpha interferon treatment.

Present results with ouabain and our previous studies with channel blocking drugs (2), indicate that the early membrane potential changes induced by alpha interferons are related to [K+] flux changes. The biological significance of [K+] flux changes through the plasma membrane is well documented. Alteration of [K+] fluxes across the plasma membrane of lymphocytes is the earliest event related to the function of the immunosuppressive agent cyclosporin A (15) and [K+] channel blockers selectively inhibit IL-2 mRNA formation (9) and prevent functions of NK cells (63). Signaling through opiate receptors (50) and serotonin receptors (13) also is intimately connected to modulation of [K+] fluxes. Thus, the alpha interferon effect is consistent with that of several other potent cytokines or bioregulators.

The (D) values Daudi cell surface antigens tested by FRAP in our experiments were comparable to data previously published for lymphocytes (5,16) and fell into an "intermediate" range (46). As shown in Table 1, the extreme molecular weights for complexes of plasma membrane antigens, antibody molecules and fluorophores in our experiments was up to threefold: i.e., the mass of anti-Leu 16/PE-B plus Leu-16 complex (ca 420,000) would be approximately one third the mass of anti-IgM-$F(ab')_2$-FITC plus sIgM complex (ca 1,300,000). In each case, however, (D) was remarkably similar. We surmise that the observed increase in mobility of sIgM complexed with FITC-Fab' fragments, as compared to FITC-$F(ab')_2$ antibody (Table 1), is more likely related to reduced cross-linking between sIgM molecules labelled with an Fab' fragment (5) than to differences in the total weight of the antigen/antibody complexes (70). Macromolecular cross-linking (78) and increased membrane occupancy (29) are factors in retarding lateral mobility and Con A is known to restrict lateral mobility both of surface lectin receptors (47,78) and immunoglobulins (29). The latter evidently reflects interactions propagated through the cytoskeleton (29). Although relatively small, the effects of Con A noted in our experiments were reproducible and in a consistently opposite direction of IFN-α2 (Table 1).

The increased (D) of several plasma membrane antigens immediately after IFN-α2 treatment was reproducible and indicates a swift reduction of protein mobility constraints. While several previous reports, including studies of cell fusion and of capping of sIgM have suggested a "decreased fluidity" of the plasma membrane after interferon treatment (8,12,53,55,58), the latter observations are not necessarily inconsistent with our FRAP results. At least two interdependent levels of molecular constraint must be distinguished: a) effects of ligands on the cytoskeleton or on receptor-cytoskeletal intractions (1,55) and b) direct effects on the surface membrane molecular environment (28,39,45). In other systems, lateral diffusion was influenced by steric or charge interactions (22,29,39,60) and recent FRAP studies indicate that configuration of surface proteins in the ectodomain (43,56,62,70) and distribution of macroscopic domains (77) may be interactive determinants of (D).

In Daudi-IFr cells, the resistance to the growth inhibitory effect of alpha interferons was associated with an increased lateral diffusion, that is the same direction as found in the IFN-α2-treated Daudi-IFs cells. Furthermore, the results indicated an "imprint" of alpha interferon, even when Daudi-IFr cells were maintained in medium without interferon. While these parallel findings are not yet explained, they do suggest that

resistance to IFN-α is entirely or partially controlled by modulation of early signal transduction with a consequent alteration of the physiologic response (36).

In view of the effects of IFN-α2 on [K+] flux discussed above and earlier observations of an altered electrophoretic charge on interferon-treated mouse cells (39), the IFN-α2-induced changes in lateral diffusion rates could be related to an altered transmembrane potential or ion flux. It must be recognized that the effects of such plasma membrane biophysical changes on membrane associated protein configuration or functional activity may be complex (27), so that the results of our experimental measurements with IFN-α2 cannot yet be definitively interpreted at a mechanistic level; nevertheless, the possibility that accelerated diffusion of interferon receptors may promote physiologic linkage to immobile [K+] channels (cf.34,67) is most intriguing.

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Discussion with Reviewers

Reviewer III: It is extremely important that the investigators demonstrate that geometric influences are not reflected in the small differences they observe following IFN-α2 treatment.
Authors: During the short exposures to IFN-α2, cell size does not change significantly, as shown by comparing the 90° light scatter in flow cytometric studies (text reference 28). Only longer exposures to IFN-α influence the cell and cell sizes (text reference 64), and must be compared to other controls for these effects. Surface ruffling of neoplastic lymphocytes treated by IFN-α has been reported (Gamliel H, Hiraoka A, Golomb HM, The effect of cultivation and interferon treatment on the surface morphology of hairy cell leukemia cells, Cancer Invest. **5**, 389–399, 1987), but topology may reflect as well as determine changes in the fluid-molecular dynamics (27). The 2–3 fold differences in (D) values between IFN-α2 and control cells were statistically significant. (Text table 1) and consistently increased with each probe. Similarly small differences have been noted in other systems (Magnusson K-E, Wojcieszyn J, Dahlgren C, Stendahl O, Sundquist T, Jacobson K, Lateral diffusion of wheat germ agglutinin-labelled glycoconjugates in the membrane of differentiating HL-60 and U-937 cells assessed with fluorescence recovery after photobleaching, Cell Biophys. **5,** 119–128, 1983; and Yechiel E, Barenholz Y, Henis YI, Lateral mobility and organization of phospholipids and proteins in rat myocyte membranes. Effects of aging and manipulation of lipid composition, J. Biol. Chem. **260,** 9132–9136, 1985). The ultimate test of biologic significance lies in the comparison of Daudi-IFs and Daudi-IFr. These cells have similar doubling kinetics and cannot be distinguished by size during log-log growth.

C.F. Hazlewood: In view of our knowledge of transmembrane proteins with intracellular anchoring as well as the significant protein associations with surface proteins, how can we be certain that the FRAP experiments measure lateral diffusion? Is this true diffusion or simply the rearrangement of proteins with intracellular connections?
Authors: These basic questions regarding FRAP measurements have been addressed in many experiments (text references 31, 33, 42–45, 56, 62). Constraints upon free lateral diffusion of proteins in the surface membrane appear to be governed primarily by factors in the ectodomain rather than by any intracellular connections or energy-dependent anchorage modulation.

H. Gamliel: The results are intriguing, but it is not clear enough as to why the observed increase in lateral diffusion of membrane molecules does not contradict other reports on decreased fluidity of the membrane and impairment of receptor rearrangement after interferon.
Authors: Several important differences must be considered: 1) Contraction of the cytoskeleton or membrane movements can be directly involved in the capping process (Schroit AJ, Pagano RE, Capping of a phospholipid analog in the plasma membrane of lymphocytes, Cell **23,** 105–112, 1981), whereas lateral diffusion can be independent of anchorage modulation (text references 42–45, 62). 2) Our experiments measured changes at <30 min compared to some previous work which showed impairment of capping at 3 to 6 h (text reference 55). 3) Although IFN-α eventually does apear to increase cytoskeletal rigidity (text reference 52), our experiments now show that IFN-α2 does not act like Con A on lateral diffusion constraints: Con A can cross-link receptors to the cytoskeleton and cause secondary effects on adjacent molecules (text references 29, 78). 4) Electron spin resonance measurements have suggested decreased fluidity of the lipid phase in interferon-treated cells (text reference 53), but this technique measures rotational movements of an introduced probe rather than lateral translation of endogenous membrane proteins. The membrane molecular environment is very complex (text references 33, 45, 60) and FRAP measures only one dimension of the total dynamics (see Lands WEM, Davis FS, Definitions, explanations, and an overview of membrane fluidity, In Aloia RC (ed.), Membrane Fluidity in Biology, Vol. 2. Academic Press, Orlando, FL., pp. 1–4, 1983). Previous studies have illustrated that diffusion of lipids and proteins may be independently modulated (text reference 56, De Laat SW, Van der Saag PT, Elson EL, Schlessinger J, Lateral diffusion of membrane lipids and proteins during the cell cycle of neuroblastoma cells. Proc. Natl Acad. Sci. USA **77**, 1526–1528, 1980).

Reviewer III: Modulation of lateral diffusion following Con A addition did not reflect the magnitude of changes observed by others, namely 5 to 10-fold decreases in lateral mobility.
Authors: This is true. We cannot explain why greater quantitative differences were not observed, even with high concentrations of Con A (100 μg/ml), but differences in anchorage modulation have been noted by others (text reference 78). Since Daudi cells are neoplastic, the cytoskeletal matrix may be differently configured than in normal lymphocytes which respond more dramatically (text reference 29). More recently we increased the Con A incubation time from 5–10 min to 30 min. We then obtained up to 3-fold differences after IFN-α2 treatment.

H. Gamliel: Since cells undergoing antibody-antigen reactions or receptor-ligand conjugation tend to rearrange their surface components (i.e., patching, capping), don't you agree that these events preceding IFN-α2 treatment could impact the reliability of subsequent measurements? How can you relate the observations to IFN-α2 itself rather than to IFN-α2 plus events triggered beforehand?
Authors: Because it is essential to label proteins with chromogenic ligands for FRAP measurements, this technique obviously cannot determine how the presence of a labeled probe *per se* alters the intrinsic diffusion coefficient of a given surface antigen. For this reason FRAP data can only be interpreted relative to other biologic systems and controls. We labelled the cells at 4°C, then added IFN-α2 at ambient temperature. During a limited time of examination (30 min) more than 90% of cells showed a ring fluorescence. There was only minimal patching in scattered cells, and FRAP measurements could be repeated on the same cell without induction of capping.

H. Gamliel: Sodium azide is a well known stabilizer of membrane components, utilized for preventing rearrangement of the surface receptors following interaction with ligands. How then could you correctly determine the effect of IFN-α2 on your measurements?
Authors: As discussed above, the relation of lateral diffusion measurements to capping and patching dynamics is complex. In contrast to capping, lateral diffusion measured at room temperature or in the presence of Na azide is not energy dependent and does not involve cytoskeletal propagation (see text references 16, 29, 45, 56, 62).

H. Gamliel: Most of your measurements were done on cells which were first labeled and then treated with IFN-α2. This is different from other systems where analysis was done on cells which are first treated with IFN and then labelled or stained. Why did you prefer labelling cells with antibody prior to incubation with IFN-α2?
Authors: We chose to pre-label, since the effect of IFN-α2 on membrane dynamic occurs within 10 min and the antibody labelling steps, including washes, require >30 min.

H. Gamliel: It is not clear why probes for Leu-12 and Leu-16 were selected for these studies or why reagents against receptors other than IgM were not tested.
Authors: Reactions of a number of commercially available chromophore-labelled monospecific or monoclonal antisera with Daudi cells were tested. Of the FITC-coupled reagents, only anti-IgM produced a sufficient intensity for FRAP analyses; however, some commercially available PE-conjugates afforded higher fluorescent yields. We empirically chose Leu 12 and Leu 16 surface antigens which differ in molecular weights; however, preliminary tests with anti-Leu 8 and anti-CALLA gave similar (D) values. Daudi cells lack surface HLA antigens and beta microglobulin.

H. Gamliel: In order to enhance labeling with antibodies, researchers prefer the indirect labeling methods which amplify the staining intensity. Could you elaborate on what might be the extent of differences in measurements if receptors/antigens are labelled indirectly, i.e., by using primary antibody followed by FITC-tagged secondary antibody? Do such longer complexes resemble IgM in their lateral diffusion coefficients?
Authors: Findings in text reference 5 indicate that the effect of size of a given receptor antibody complex on the diffusion coefficient appears to be less than the effects of cross-linking between surface molecules. Our data with PE-B label confirms that relatively large probe-protein complexes can diffuse in the range of 5×10^{-10}cm^2/sec. Indirect labelling would probably decrease lateral diffusion only to the extent that cross-linking were introduced.

C.F. Hazlewood: I am quite certain that the dye used in this paper is in some way related to the cellular potential; however most dyes used to determine the "membrane potential" are bound to cellular constituents (see Wilson HA, Seligmann BE, Chused

TM, Voltage sensitive cyanine dye fluorescence signals in lymphocytes: plasma membrane and mitochondrial components. J. Cell Physiol., **125,** 61–71, 1985). How, therefore can one use the Nernst equation which is derived from dilute solution theory (73) to estimate membrane potential? Please also refer to Negendank W, Ion and water retention by permeabilized cells, Biochim. Biophys. Acta, **694,** 123–161, 1982 and Ling, GN, Electrical potentials, In: In Search of the Physical Basis of Life, Plenum Press, pp. 477–494, 1984.

Authors: The oxonol dye which we used was introduced as a superior method for detecting membrane potential (text reference 73). Such dyes do dissolve into the membrane lipid domain and therefore they truly reflect the [K+]-driven membrane potential which can be described by the Nernst equation. Related references on the use of these dyes for membrane potential measurements include: Waggoner AS, Wang CH, Tolles RL, Mechanism of potential-dependent light absorption changes of lipid bilayer membranes in the presence of cyanine and oxonol dyes. J. Membr. Biol., **33,** 109–140, 1977; Rink TF, Montecucco C, Hesketh TR, Tsien RY, Lymphocyte membrane potential assessed with fluorescent probes, Biochem. Biophys. Acta, **595,** 15–30, 1980; Brauner T, Hulsere DF, Strasser RJ, Comparative measurements of membrane potentials with microelectrodes and voltage-sensitive dyes, Biochim. Biophys. Acta, **771,** 208–246, 1984.

C.F. Hazlewood: The effect of IFN-α2 on the cellular potential (Fig. 1) is remarkable and most interesting. Is it possible that the potential is a surface adsorption potential rather than a membrane potential? The use of some surface or phase boundary theory might be helpful in overcoming the problems of dye adsorption within the cell.

Authors: We recognize that different intracellular compartments (e.g., Hazlewood CF, Kellermayer M, Ion and water retention by permeabilized cells, Scanning Microsc. **2,** 257–273, 1988) may have their own potentials which are distinct from the plasma membrane potential; however, oxonol dye measurements do not reflect any potential, other than that of the plasma membrane (text reference 73). In lymphocytes only [K+] fluxes influence membrane potential in the range which we measure (i.e., circa −55 mV). Thus, our data are logically interpreted as reflecting the effect of IFN-α2 binding on transmembrane [K+] fluxes.