In Vitro Cell. Dev. Biol. 27A:633–638, August 1991

0883-8364/91 $01.50+0.00

TOXICITY IN VITAL FLUORESCENCE MICROSCOPY: EFFECT OF DIMETHYLSULFOXIDE, RHODAMINE-123, AND DiI-LOW DENSITY LIPOPROTEIN ON FIBROBLAST GROWTH IN VITRO

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(Received 25 September 1990; accepted 18 March 1991)

SUMMARY

Fluorescence microscopy performed on living cells is a valuable technique for elucidating patterns of cell growth in vitro over artificial biomaterials such as vascular grafts, and for in vivo studies such as identification and treatment of atherosclerotic plaques. Two fluorescent dyes of particular value for vital fluorescence studies are Rhodamine-123 and 3,3'-dioctadecylindocarbocyanine-labeled low density lipoprotein (DiI-LDL). We examined the toxicity of these two dyes and of dimethylsulfoxide (DMSO), a solvent used in Rhodamine-123 studies, on the growth of MRC[5] human fetal fibroblasts in monolayer culture. Two parameters of cell growth were quantitated: Cell number (a measure of proliferation), and cell area (a measure of cell spreading), based on microscopic images obtained at the start and end of a 48-h growth period after brief exposure (0.5 h) to test solutions. We found that the recommended solvent for solubilization of Rhodamine-123, DMSO, caused cessation of cell proliferation and actual reduction in the area covered by adherent fibroblasts at concentrations of as low as 0.1% (vol:vol). Rhodamine-123 made up from an aqueous stock solution modestly retarded proliferation and spreading, and there was no significant effect of DiI-LDL on these parameters over prolonged periods of exposure (up to 24 h) in culture. These results demonstrate that the most toxic substance for growing fibroblasts was the solvent DMSO. We conclude that both the solvent vehicle and fluorescent dye should be carefully examined for potential toxicity before such dyes are used for vital fluorescence studies of living cells.

Key words: fibroblast; fluorescence microscopy; dimethylsulfoxide; Rhodamine-123; DiI-Low density lipoprotein; vital staining.

INTRODUCTION

The use of vital fluorescent stains to label living cells grown in culture has become a major tool of modern cellular biology (Arndt-Jovin et al., 1985; Taylor and Wang, 1980). However, evaluation of the toxicity of such fluorescent probes is a critical initial step for such investigations. In addition, fluorescent probes may have therapeutic value, such as in labeling atherosclerotic plaques for laser ablation (Andersson-Engels et al., 1990, Grundfest et al., 1985). The clinical utility of labeling protocols will be limited, however, if the fluorescent probes prove toxic to endogenous tissues such as the cells lining vascular channels.

Published protocols for staining living cells with fluorescent probes routinely call for 30-min to 1-h exposures to medium containing a probe made up from a concentrated stock solution in dimethylsulfoxide (DMSO), for example the cationic mitochondrial stain Rhodamine-123 (Goldstein and Korczack, 1981). Up to 24 h are allowed for uptake of a fluorescent lipoprotein derivative, 3,3'-dioctadecylindocarbocyanine-labeled low density lipoprotein (DiI-LDL) by living cells (Jaakkola et al., 1988; Kingsley and Kreiger, 1984; Kreiger et al., 1979). Early in our studies of vital fluorescent staining of cells in vitro (Crawford et al., 1989), we found that cell growth in tissue culture after exposure to a vital stain was erratic, and outright cell death was observed after brief exposure to some fluorescent cocktails. Such toxic effects would confound basic investigative studies and raise clinical concern about potentially harmful effects of staining reagents on endogenous tissues. We therefore undertook a systematic study of the effect of Rhodamine-123 and DiI-LDL on human fetal fibroblasts in culture during their linear phase of growth.

Growth was measured by quantitating two parameters on digitized microscopic images: the proliferation of fibroblasts, using cell counts; and the progressive spreading of adherent fibroblasts over the tissue culture plates, using morphometric analysis of cell area. We found that the most toxic substance to both fibroblast proliferation and spreading was the DMSO used to dissolve Rhodamine-123. Rhodamine-123 derived from an aqueous stock solution exerted a modest but significant retarding effect on these two parameters, and there was no significant alteration in cell proliferation or spreading during prolonged exposure to DiI-LDL.

MATERIALS AND METHODS

Materials. Rhodamine-123 (Molecular probes, Eugene, OR) and DiI-low density lipoprotein (Biomedical Technologies, Stoughton, MA) were obtained commercially. Earle's minimal essential medium (EMEM) and fetal bovine serum were obtained from GIBCO, Inc. (Grand Island, NY). Presterilized DMSO was from American Type Culture Collection (Rockville, MD). All other reagents were of the highest grade available commercially.

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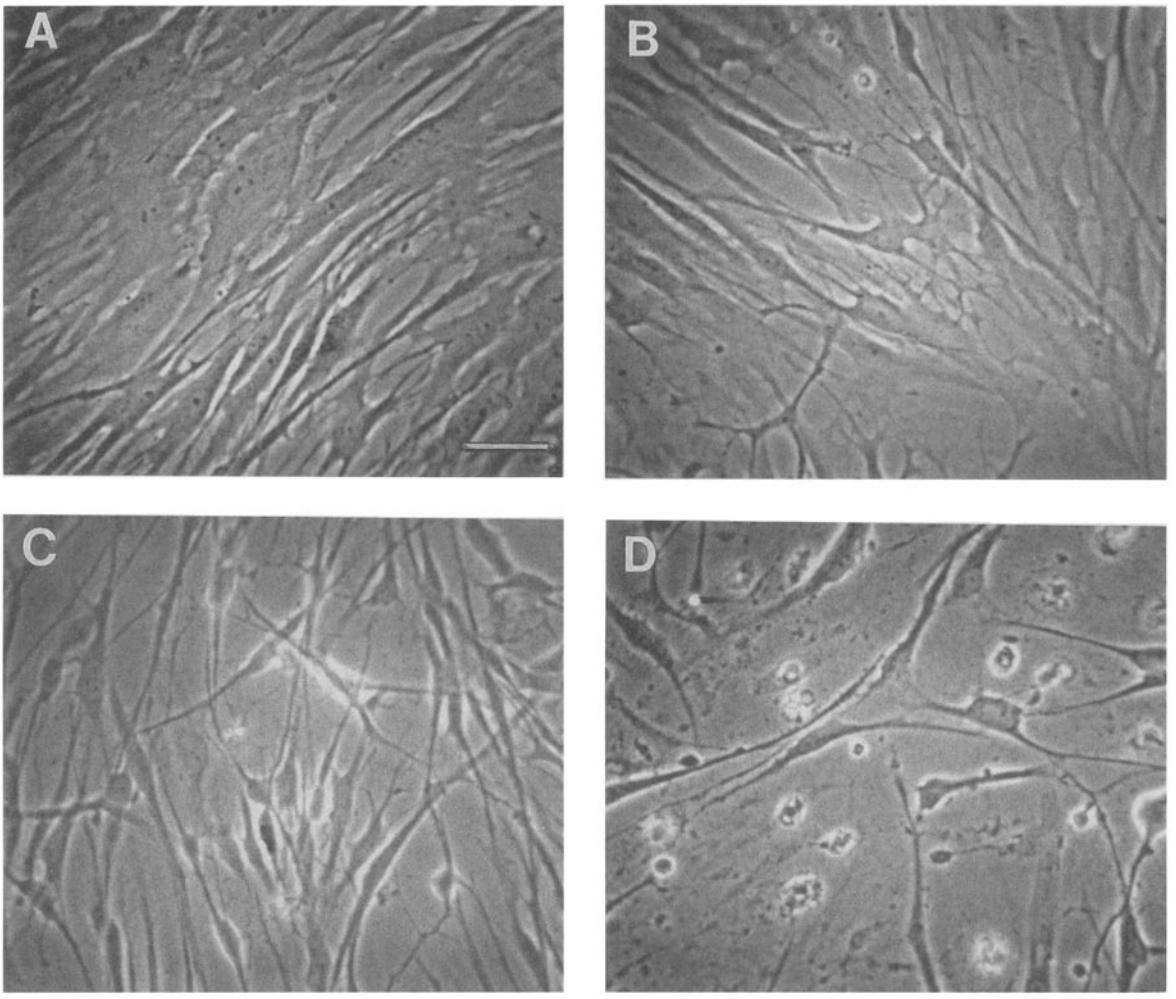

FIG. 1. Morphology of fibroblasts 48 h after 0.5 h of exposure to DMSO. Fibroblasts were exposed to increasing concentrations of DMSO (%, vol:vol) for 0.5 h, rinsed, and grown in culture for 48 h (*see* Methods). Progressive dimunition in cell growth is evident after exposure to DMSO concentrations of zero (*A*), 0.1% (*B*), 0.5% (*C*), and 2.5% (*D*). Phase contrast microscopy, *Bar* = 25 μm.

Preparation of test solutions. A stock solution of Rhodamine-123 (received as a dry powder) in DMSO was prepared directly (1 mg/ml; Goldstein & Korczack, 1981); no further sterilization was performed. Stock solutions of Rhodamine-123 in water (1 mg/ml) were prepared using double distilled water, and sterilized by filtration using a Milex 0.22 μm filter (Millipore Corp., Bedford, MA). Acetylated low density lipoprotein labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI-LDL) was received as a sterile solution (200 μg/ml) in 150 m*M* NaCl, 50 m*M* tris (pH 7,4), 2 m*M* ethylenediaminetetraacetic acid (EDTA).

Cell culture. Human fetal fibroblasts (MRC^5 cells) were obtained commercially from Whittaker Bioproducts (Walkersville, MD) as a suspension in EMEM supplemented with 10% fetal bovine serum. Suspended cells (5×10^5) in 2 ml of medium were plated in 25-cm^2 Falcon tissue culture flasks and allowed to adhere for 24 h in a 5% CO_2 atmosphere in a 37° C incubator (adherence period). The medium was then changed to 2 ml of EMEM (GIBCO) containing appropriate concentrations of DMSO or fluorescent probes. After further incubation at 37° C for specified time periods (exposure period), the flasks were rinsed twice with 2 ml EMEM, 2 ml fresh EMEM were added, and flasks were returned to the incubator for a 48-h growth period. Preliminary experiments showed that cell growth was comparable when 2 or 5 ml of medium was added, and that fetal bovine serum was not required for adequate cell growth over 96 h. To minimize the potential influence of contaminating growth factors, experimental culture media did not contain fetal bovine serum.

Fluorescence microscopy and digital image analysis. Phase and epifluorescence microscopy were performed using the 40× objective of a Nikon-Diaphot inverted microscope. A B-2A Nikon fluorescent filter assembly and a 100-W mercury light source were used for epifluorescence illumination, with a no.16 neutral density filter (6.25% transmission of light) in the light path. Video images on four randomly selected fields from each flask for each time point were obtained with a Hamamatsu C2400-08 Silicon-Intensified Tube video camera, and captured using an IBM-compatible 386 computer containing Data Translation image capture and co-processor boards (Marlboro, MA). To enhance clarity of each image, eight successive images were frame-averaged (elapsed time less than 1 s). Morphometric analysis of cell area in each image (in pixels) was

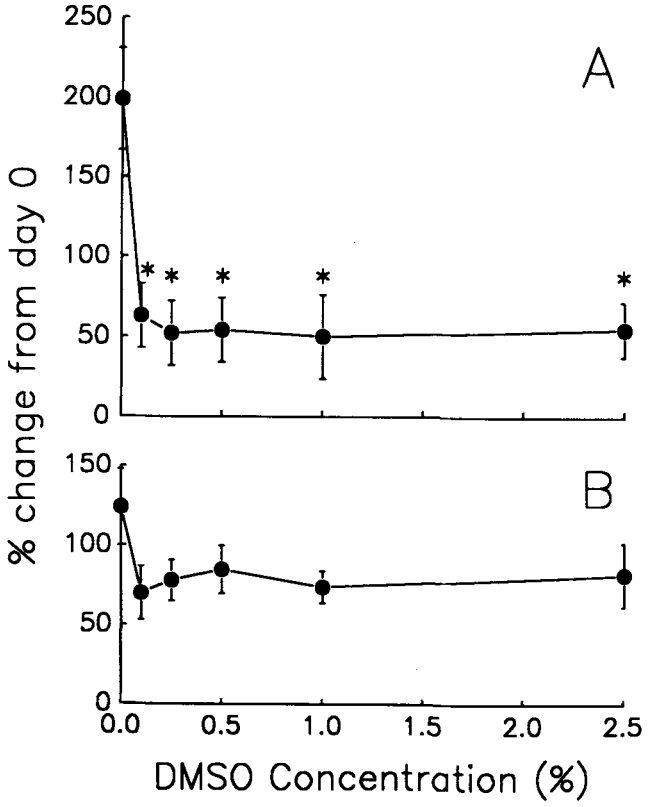

FIG. 2. Effect of DMSO on fibroblast growth parameters. Fibroblast growth after brief exposure to DMSO-containing solutions (0.5 h) is expressed as the percent change in two parameters of cell growth. *A*, change in cell coverage of the plate, based on morphometric measurement of the change in cell area from the beginning (100%, calculated on a per plate basis) to the end of the 48-h growth period (*see* Methods). *B*, change in cell count per video field over the same period. *Asterisk* = $P < 0.01$ relative to control. (mean ± SEM, $n = 4$ images per flask at start and end of growth period, one flask per condition in four experimental series).

performed using Image-Pro software (Media Cybernetics, Silver Spring, MD) and Image Measure module 4500 (Microscience, Federal Way, WA). Cell number was assessed by counting individual cells in each image: cell counts above 20 were not considered reliable due to the difficulty in distinguishing individual cells in subconfluent cultures.

Each experimental measurement consisted of four randomly selected images from each flask for each time point, from which mean values for cell count and area were obtained for the beginning and end of the 48-h growth period, allowing the computation of percent increase in these parameters for each flask. Individual experiments consisted of a complete series of flasks exposed to varying concentrations of DMSO or Rhodamine-123 for 0.5 h before a 48-h growth period (sets of six flasks each), or three sets of five flasks exposed to increasing concentrations of DiI-LDL for 0.5, 6, or 24 h during the 48-h growth period. Each individual experiment was repeated independently, using different batches of cells, DMSO, and fluorescent labels ($n = 4$ for the DMSO/Rhodamine-123 experiments, and $n = 3$ for the DiI-LDL experiments), allowing the computation of mean ± SEM of the percent increase in cell count and area after exposure to each test solution. Excluding preliminary work, a total of 744 video images on 93 flasks from 7 experiments were analyzed in this study.

Statistical methods. Statistical comparisons were performed using analysis of variance followed by Neuman-Keuls multiple range tests for pairwise comparisons (Zar, 1974).

RESULTS

After the 24-h adherence period after plating of 5×10^5 fibroblasts in 2 ml of medium, 30.1 ± 5.6% of the tissue culture flask was covered by adherent cells, with 10.3 ± 1.7 cells per video field (mean ± SEM, $n = 7$ batches of cells). The exposure periods were chosen based on published protocols for vital staining of cell cultures. A 48-h growth period was selected after preliminary experiments showing optimal reproducibility in measured cell proliferation and spreading; differences between treatment groups were not reliably evident at 24 h, and cultures had frequently grown to confluence by 72 h.

Effect of DMSO on fibroblast growth. Our initial experiments were directed at examining the effect of Rhodamine-123 on fibroblast growth, using a standard protocol which involved dilution of a stock solution in DMSO with tissue culture medium (Goldstein and Korczack, 1981). Preliminary results suggested that the solvent exerted a primary toxic effect; no cells remained after a 24-h incubation at 37° C with medium containing 0.25% DMSO or greater. Fibroblasts were therefore exposed to increasing concentrations of DMSO for 0.5 h (exposure period). Figure 1 shows the morphologic features of fibroblasts after 48 h subsequent growth in fresh medium. In control cells not exposed to DMSO (*panel A*), subconfluence was obtained, with the fibroblasts demonstrating their characteristic elongate shape. Cell growth was severely impaired by exposure to DMSO as shown in *panels B–D*, which illustrate progressive decrease in cell number and attenuation of cell area (actinomorphic change), with accumulation of cell debris in *panel D*. Figure 2 shows the percent change in cell area over the 48-h growth period after exposure to increasing concentrations of DMSO (*panel A*), and the corresponding percent change in cell number (*panel B*). Surprisingly, even the lowest tested concentration of DMSO, 0.1% (vol:vol), caused a substantial reduction in cellular area ($P < 0.05$) as well as a reduction in cell count (not significant). To exclude the possibility that DMSO was leaching toxic chemicals from the plastic culture falsks, these experiments were repeated using glass incubation dishes, with similar results.

Effect of Rhodamine-123 on fibroblast growth. After specific examination of the toxicity of DMSO (*see* above), an aqueous stock solution of Rhodamine-123 was prepared. Fluorescent labeling of fibroblasts occurred within 0.5 h of exposure to Rhodamine-123 (10 μg/ml; data not shown); cells remained viable and fluorescent over 24 h of exposure to this concentration of Rhodamine-123, as shown in Fig. 3, *panels A and B*, confirmed by fluorescein diacetate uptake (Crawford et al., 1989). However, fibroblast growth was modestly retarded by Rhodamine-123, as shown in Fig. 4, which records the change in cell area (*panel A*, $P < 0.05$) and cell count (*panel B*, not significant) over the 48-h following 0.5h exposure to increasing concentrations of this probe. To determine whether Rhodamine-123 caused outright cell death over longer periods of exposure, tissue culture flasks with fibroblasts grown to confluence were incubated with medium containing Rhodamine-123 (2.5 to 50 μg/ml) for 0.5, 6, and 24 h, followed by rinsing twice with medium and

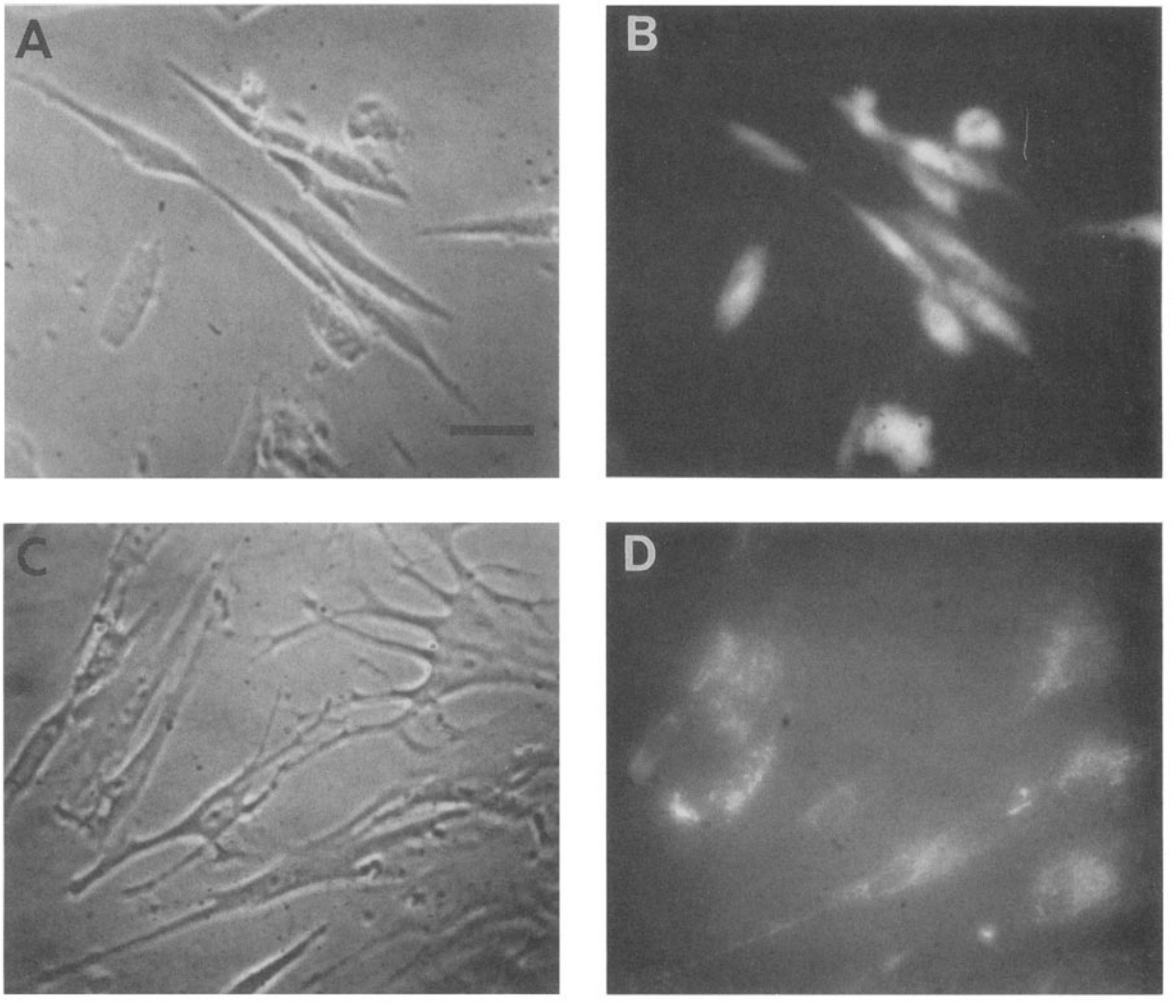

FIG. 3. Uptake of Rhodamine-123 and DiI-LDL by growing fibroblasts. Fibroblast fluorescence after 24 h of exposure to Rhodamine-123 (10 μg/ml, made up from 1 mg/ml aqueous stock; *A* and *B*) or 48 h exposure to DiI-LDL (10 μg/ml, made up from 200 μg/ml aqueous stock; *C* and *D*) is illustrated. Phase contrast microscopy (*A* and *C*), epifluorescence microscopy (*B* and *D*). *Bar* = 25 μm.

further incubation for observation at 24 h. All flasks remained confluent, with no evidence of cell lysis or loss of adhesion.

Effect of DiI-LDL on fibroblast growth. A similar protocol was employed for documenting the effect of fluorescently labeled LDL on cell growth. However, inasmuch as published protocols for labeling of cells with DiI-LDL describe incubations of up to 3 to 5 h (Kingsley and Kreiger, 1984), we chose to expose growing fibroblasts to fluorescent medium for 0.5, 6, and 24 h, and include these exposure periods in the "growth period." Satisfactory labeling was achieved even after 48 h, as shown in Fig. 4, *panels C and D*. Unlike DMSO and Rhodamine-123, DiI-LDL had no significant effect on fibroblast growth for any of these exposure periods, with flasks showing a doubling in cell area, and a 65% increase in cell count over the 48-h growth period.

DISCUSSION

Recent advances in the development of fluorescent-labeled probes have created new opportunities to study living cells as they grow and interact with one another. Our particular area of interest is the in vitro neovascularization of biosynthetic devices that may be implanted in the circulation, such as arterial grafts and heart valves (Brais and Braunwald, 1974; Braunwald, 1989). The relationship between cellular components of the arterial wall and artificial surfaces can now be examined by fluorescence microscopy, using undisturbed, living cells in tissue culture (Crawford et al., 1989). In addition, the recent use of laser catheterization to ablate atherosclerotic lesions has generated considerable interest in methods to precisely localize target areas within the arterial wall. The potential for specifically labeling atherosclerotic lesions is sparked in part by recent evidence that they may be amenable to fluorescent labeling. For example, DiI-LDL and its acetylated derivative are actively taken up by foam cells (macrophages) and smooth muscle cells derived from atherosclerotic lesions (Jaakkola et al., 1988). Thus, externally applied fluorescent probes might increase the sensitivity and accuracy of efforts to identify lesional tissue within blood vessels (Kingsley and Kreiger, 1984; Prevosti et al., 1988).

Previous studies of cell growth on defined matrices have involved

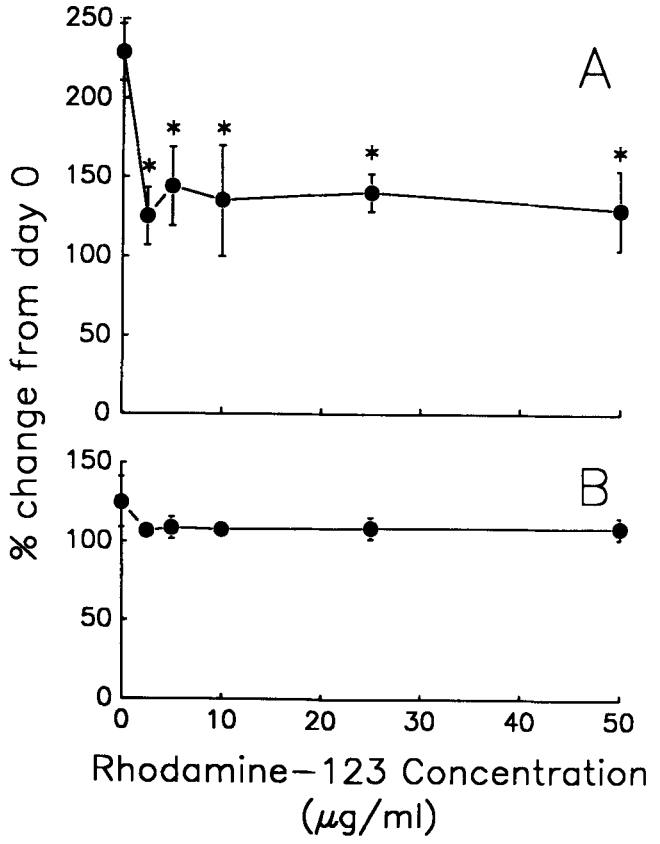

FIG. 4. Effect of Rhodamine-123 on fibroblast growth parameters. Fibroblast growth after 0.5 h of exposure to increasing concentrations of Rhodamine-123 (made up from 1 mg/ml aqueous stock) is expressed as percent change in cell area and in cell count over the 48-h growth period (*see* Methods and Fig. 2). *Asterisk* = $P < 0.05$ relative to control. (mean ± SEM, $n = 4$ images per flask at start and end of growth period, one flask per condition in four experimental series).

fixation of cells before microscopic examination (Madri and Stenn, 1982; Anderson et al., 1985). Trypsinization of adherent cells after a growth period permits assessment of the number of cells released into suspension (Metcalfe, 1971). Unfortunately, both methods eliminate the possibility of continually monitoring cell growth. Although observation of living cells in culture by light microscopy is not disruptive, cell numbers alone do not provide adequate information on the adequacy of surface coverage. This is illustrated by the images of Fig. 1 and by the minimal changes in cell counts shown in *panel B* of Figs. 2 and 4. Indeed, rounding up or actinomorphic changes in cultured cells is a standard method for assessing the presence of bacterial toxins in biological specimens (Chang et al., 1979). Rather, the ability of fibroblasts to remain flattened and adherent to a tissue culture surface is an important measure of viability (Hayflick, 1977). The morphometric technique presented in this study was chosen to obtain reliable and reproducible assessment of cellular spreading and integrity in situ, based on quantifying the area coverage of a tissue culture flask by living cells growing in cell culture (*panel A* of Figs. 2 and 4).

The digital images in Fig. 1 illustrate the spectrum of morphologic appearances of fibroblasts 48 h after a brief (0.5 h) exposure to DMSO at low concentrations. Figure 2 demonstrates that exposure to even the lowest concentration of DMSO (0.1%) at 37° C leads to a marked reduction in cell spreading over the ensuing 48 h. This effect was observed using different batches of DMSO and with glass culture dishes, so is not likely to be due to the presence of contaminants in the DMSO or leaching of materials from the plastic dishes. Moreover, it is unlikely that further toxins were generated in our hands, because no further sterilization of the Rhodamine/DMSO stock solution was needed before use. Our finding is at variance with reports that cell cultures grown in the presence of 1.0% DMSO did not differ in growth rate from control cells (Diamond, 1965; Metcalfe, 1971), although inhibition of cell growth has been noted at higher concentrations (Collins et al., 1978; Hellman et al., 1967). These earlier studies, however, were based on cell counts after trypsinization of growing cells, rather than our method of direct microscopic observation. To the extent that fibroblast adherence and spreading over a tissue culture flask represents normal cell behavior, our studies indicate that DMSO is a markedly disruptive agent. Corroborating this observation, toxicity of DMSO to nonproliferating tissues has been repeatedly observed, particularly at exposure temperatures of 37° C (Hak, 1973; O'Neill et al., 1967; Penninck et al., 1983).

The lipophilic cationic dye Rhodamine-123 is taken up by living cells and specifically concentrated in mitochondria (Johnson et al., 1981), and may be selectively toxic for cancer cells in vitro (Goffney et al., 1990; Krag et al., 1990), possibly by inhibition of mitochondrial energy production (Bernal et al., 1983). Toxicity of Rhodamine-123 has been documented in Friend leukemia cells at concentrations of as low as 0.10 μg/ml over a 72 h in culture (Lampidis et al., 1989). Johnson et al. (1980) reported no effect of aqueous-based Rhodamine-123 on growth rate of living gerbil fibroma cells over 96 h in culture; however, the method of quantitation was not reported. We have now documented that this fluorescent probe may modestly retard fibroblast spreading, with no significant decrease in proliferation (Fig. 4) and no apparent loss in cell viability over long periods of exposure (Crawford et al., 1989, *see* Results). Although these results are not as dramatic as those observed with malignant cells (Bernal et al., 1982; Krag et al., 1990), they nevertheless illustrate the need for care in studying cell growth in the presence of this dye.

Fluorescently labeled low density lipoprotein (DiI-LDL) was included in this study because of its potential use in basic investigation and treatment of atherosclerotic lesions. Unlike Rhodamine-123, which may affect mitochondrial function and thereby cell viability (Bernal et al., 1983), the fluorescent lipid taken up by lipoprotein-metabolizing cells is directed toward endocytotic compartments, and has no measurable effect on cellular function (Kingsley and Kreiger, 1984). The staining of fibroblasts and retention of viability over 48 h of exposure (Fig. 4) and lack of effect on cell growth parameters observed in this study provide reassuring evidence the DiI-LDL has no significant toxic effect of living cells.

In summary, this report presents a methodology for quantifying growth parameters of living cells in vitro, using morphometric analysis of microscopic images to obtain direct information about the adhesion and spreading of cells over a culture substratum, in conjunction with easily obtained cell counts. This methodology has been used to examine the toxicity of two fluorescent dyes which have proven particularly useful for investigations of living cells, Rhodamine-123 and DiI-LDL. We found that the recommended solvent for solubilization of Rhodamine-123, DMSO, proved to be

the most disruptive substance, after brief exposure (0.5 h) and at concentrations of as low as 0.1% (vol:vol, equivalent to 7 m*M*). Rhodamine-123 modestly retarded cell spreading, and there was no significant effect of DiI-LDL over prolonged periods in culture. We conclude that both solvent vehicle and fluorescent dye should be carefully examined for potential toxicity before such dyes are used for vital fluorescence studies of living cells.

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This study was supported in part by grants DK-39512 and by BRSG 807 RR05950 from the National Institutes of Health, Bethesda, MD. A portion of this work was performed while J.M.C. was an American Gastroenterology Association/Searle Industries Research Scholar Award Recipient.