

PHOTOSENSITIZATION TO ULTRAVIOLET IRRADIATION AND SELECTIVE KILLING OF CELLS FOLLOWING UPTAKE OF PYRENE-CONTAINING FATTY ACID

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SUMMARY

Cells were incubated with 12-(1-pyrene)-dodecanoic acid (P12), a long-chain fatty acid to which a pyrene ring has been attached covalently. This acid was transported across the plasma membranes of cells and subsequently incorporated into their neutral lipids and phospholipids. Irradiation of these pyrene-containing cells for short periods (0.5–4 min) with ultraviolet light at 366 nm resulted in eventual cell death. Similar irradiation had no effect on cells that had not been exposed to P12. The time of the period of irradiation necessary for inducing the toxic process was related to the quantity of P12 incorporated, the latter being a function of the respective metabolic activity of the individual cell type. The degree of incorporation of P12 into a cell, and consequently its acquired sensitivity to killing by ultraviolet irradiation at 366 nm, was affected by the incubation temperature and addition of non-fluorescent fatty acid, albumin or other serum proteins. Different degrees of incorporation of P12 into various cell types were used for selective killing and elimination of cell populations by irradiation at 366 nm. The combined procedure of preincubation with P12 followed by ultraviolet irradiation thus permitted selection of cell types with a greater resistance to this procedure.

INTRODUCTION

Fluorescent derivatives of fatty acids (FDFA) have been used for studying various aspects of fatty acid uptake (Morand *et al.* 1982), lipid–protein interaction (Stoffel & Michaelis, 1976*a,b*), lipid metabolism (Gatt *et al.* 1980), and membrane structure (Vanderkooi *et al.* 1974) and physicochemical properties (Thulborn & Sawyer, 1978).

We have studied the uptake and metabolism of FDFAs by cultured cells and developed procedures for following the uptake, as well as sorting out and separating cells according to their relative fluorescent intensities (Nahas *et al.* 1986). One FDFA, 12-(1-pyrene)-dodecanoic acid (P12), was found to be effectively transported and subsequently incorporated into cellular neutral and phospholipids

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(Morand *et al.* 1982, 1984). Differences in the rate of P12 uptake and incorporation were observed among various cell types. Cell differentiation was also associated with changes in the rate of P12 uptake and incorporation (Fibach *et al.* 1986).

Pyrene has been reported to be an efficient photosensitizing agent. Irradiation by ultraviolet (u.v.) light of cells into which pyrene-cholesteryl ester was introduced resulted in effective cell killing (Mosley *et al.* 1981). The present study made use of the differences in the rate of uptake and incorporation of a pyrene-containing fatty acid by cells of various types or cells of the same type but at various stages of differentiation, for a selective killing of cell populations on the basis of their acquired photosensitivity to u.v. light.

For this purpose, two *in vitro* established leukaemic cell lines that are blocked at early stages of maturation were used. HL-60, originally established from a patient with acute promyelocytic leukaemia (Collins *et al.* 1977), shows characteristics of malignant myeloblasts and promyelocytes in culture (Gallagher *et al.* 1979). Murine erythroleukaemia (MEL) cells are malignant proerythroblasts (Friend *et al.* 1971). Both cell types can be induced to undergo maturation by a variety of agents: HL-60 cells into mature granulocytes (Collins *et al.* 1978) and MEL cells into haemoglobin-containing normoblasts (Marks & Rifkind, 1978). Previous experiments have shown that HL-60 and MEL cells differ considerably in the rate and extent of P12 uptake, and its subsequent incorporation into cellular lipids (Nahas *et al.* unpublished). Furthermore, differentiation in both cell lines was accompanied by considerable changes in the degree of P12 uptake (Fibach *et al.* 1986).

MATERIALS AND METHODS

The human promyelocytic leukaemia (HL-60) (Collins *et al.* 1977) and the murine erythroleukaemia (MEL) (Friend *et al.* 1971) cell lines were maintained by subculturing, every 3–4 days, at a density of 2.5×10^5 and 1×10^5 cells ml^{-1} , respectively, in alpha minimal essential medium supplemented with 10% foetal calf serum (FCS) (both from Gibco, Grand Island, NY) and incubating at 37°C in humidified atmosphere of 5% CO_2 in air. Differentiation was induced in HL-60 cells by addition of 1.25% dimethylsulphoxide (DMSO), and in MEL cells by 4 mM-hexamethylene bisacetamide (HMBA; Aldrich, Milwaukee, WI) (Reuben *et al.* 1976). The percentage of differentiated, granulocyte-like HL-60 cells was determined by the nitroblue tetrazolium reduction assay (Collins *et al.* 1980) and of differentiated, haemoglobin-containing MEL cells by benzidine staining (Orkin *et al.* 1975). Cellular morphology was determined on cytospin-prepared slides stained with May-Grunwald and Giemsa. Cloning efficiency was determined in methylcellulose-containing semi-solid medium as described (Fibach *et al.* 1982).

12-(1-pyrene)-dodecanoic acid (P12) was purchased from Molecular Probes, Inc. (Junction City, OR) or, alternatively, synthesized (G. Halperin, A. Dagan & S. Gatt, unpublished data). A solution of P12 in chloroform:methanol (2:1, v/v) was evaporated under a stream of nitrogen and dissolved in DMSO to a concentration of 4 mM. A 50 μl sample of this solution was added per 1 ml FCS, mixed and incubated at 37°C for 1 h. Cells in 9 ml serum-free medium were added and incubated at 37°C for various periods as indicated in the text. Unless otherwise stated, the final concentrations in the incubation mixture were: cells, 10^6 ml^{-1} ; FCS, 10%; P12, 20 μM ; and DMSO, 0.5%. Exposure of the cells to 0.5% DMSO for the time of incubation, followed by extensive washing, had no discernible effect on cell viability, differentiation or uptake of fatty acid.

Following incubation, cells were washed three times with serum-containing medium and resuspended in the same medium. Cell suspensions (1 ml) were dispersed into 35 mm Petri dishes, which were placed 20 cm below two u.v. lamps (Desaga, Heidelberg, Germany) and irradiated for various periods. The intensity of the incident radiation was $773 \mu\text{W cm}^{-2}$, as determined by Hilger-

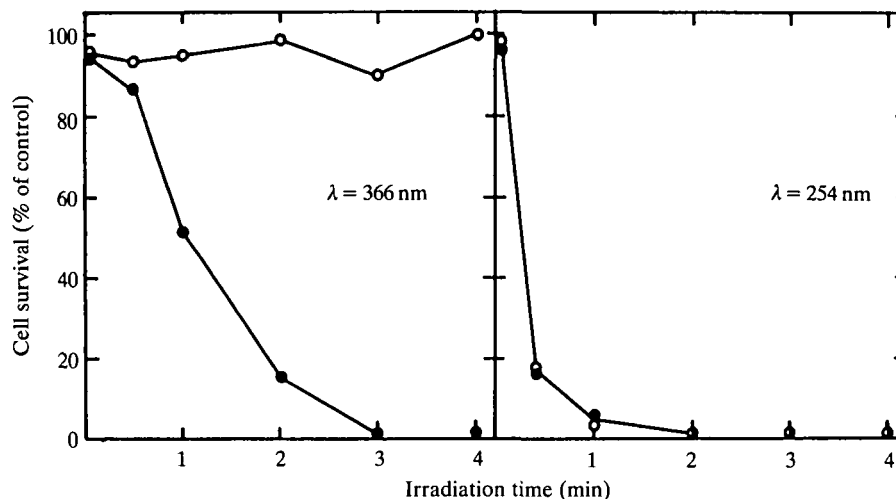


Fig. 1. The effect of u.v. irradiation on viability of P12-containing cells. HL-60 cells were incubated overnight in the presence (●) or absence (○) of $20 \mu\text{M}$ -P12, washed three times in serum-containing medium and irradiated for the indicated periods at either 366 nm or 254 nm. The cells were then incubated for an additional 24 h, and the number of viable cells was determined by Trypan Blue exclusion. Results are presented as percentage of control cultures, which were neither incubated with P12 nor irradiated.

Schwarz Thermopiles (Keithley Instruments, Munich, West Germany). After irradiation the cultures were incubated at 37°C in 5% CO_2 in air. After 16–24 h of incubation at 37°C in an incubator with 5% CO_2 in air the concentration of viable cells was determined by the Trypan Blue exclusion test. Cultures that were neither incubated with P12 nor exposed to irradiation served as controls in the experiments presented in Figs 1 and 2. Cultures incubated in the same tube with P12, but not irradiated, served as control in experiments presented in Figs 3–7. The data presented are the means of the results of four determinations in a representative experiment out of at least three experiments performed. The deviation of the results from the mean in three experiments did not exceed 20%.

Lipid extraction and analysis was performed as previously described (Morand *et al.* 1982).

RESULTS

The effect of uptake of P12 and subsequent irradiation on the viability of HL-60 cells is presented in Fig. 1. Cells were incubated overnight without or in the presence of P12, washed and then irradiated for various periods with u.v. light sources at 366 or 254 nm. The results show that irradiation at 254 nm had a considerable toxic effect, irrespective of whether the cells had been preincubated with P12. This was not the case for u.v. irradiation at 366 nm, where phototoxicity was restricted to those cells that had been preincubated with P12. In comparison, cells that were incubated with P12 but not exposed to irradiation or, alternatively, cells incubated without P12 and subsequently irradiated at 366 nm, were not affected and continued to proliferate normally.

HL-60 cells form large colonies, with high efficiency, in semi-solid medium (Fibach *et al.* 1982). Toxicity could be detected immediately following irradiation at 366 nm of P12-containing cells, by a sharp reduction in cloning efficiency; irradiation

for 4 min reduced clonability to less than 1%. Toxicity was further evident, within a few hours, by an increase in Trypan Blue positive cells and subsequently by a marked decrease in viable cell number due to cell lysis (Fig. 2).

The experiments presented in Fig. 3 tested the effects of P12 concentration (Fig. 3A) and, alternatively, the duration of exposure to one concentration of P12 (20 μM) (see Fig. 3B) on the sensitivity of the cells to irradiation at 366 nm. The photosensitization increased with increasing values of either of the two respective parameters. The results also indicate that, under the experimental conditions used, exposure to 20 μM -P12 for a period as short as 10 min was sufficient to sensitize HL-60 cells to subsequent irradiation at 366 nm. These results might be related to the fast kinetics of initial uptake of P12 as measured by spectrofluorometric and flow cytometry analyses (Morand *et al.* 1982; Nahas *et al.* 1986).

Previous experiments have shown that serum albumin decreased the rate of uptake and subsequent metabolic utilization of natural (Morand *et al.* 1983) and pyrene-containing fatty acids (Nahas *et al.* unpublished) by cultured cells. The effect of the ratio of albumin to P12 on the sensitization of HL-60 cells to irradiation is shown in Fig. 4. The data (Fig. 4A) confirm previous findings that, at a fixed concentration of P12, increasing concentrations of albumin result in less P12 uptake and incorporation into cellular neutral lipids and phospholipids. Concomitantly, a significant reduction in photosensitization ensued (Fig. 4B). Similar results were obtained when HL-60

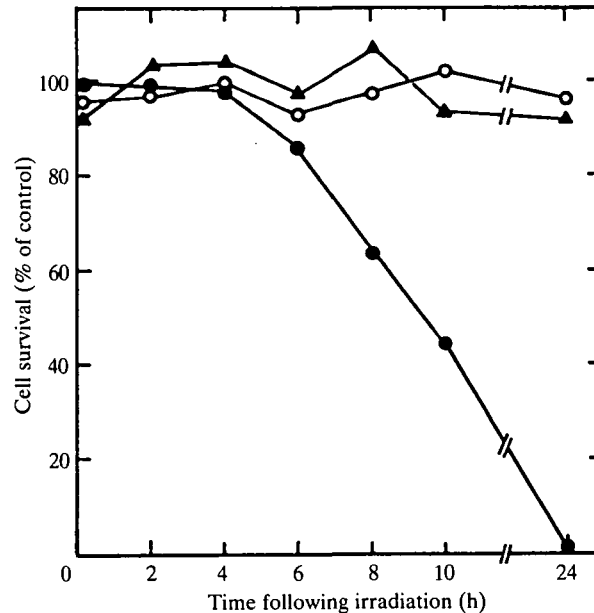


Fig. 2. Cell viability following exposure to irradiation. HL-60 cells were exposed to P12 overnight, washed, irradiated for 4 min at 366 nm and then incubated at 37°C. At various times, the concentrations of viable cells were determined following staining with Trypan Blue. Cultures that were neither exposed to P12 nor irradiated served as controls. (●) Cells preincubated with P12 and irradiated; (○) cells preincubated with P12 but not irradiated; (▲) cells preincubated without P12 and irradiated.

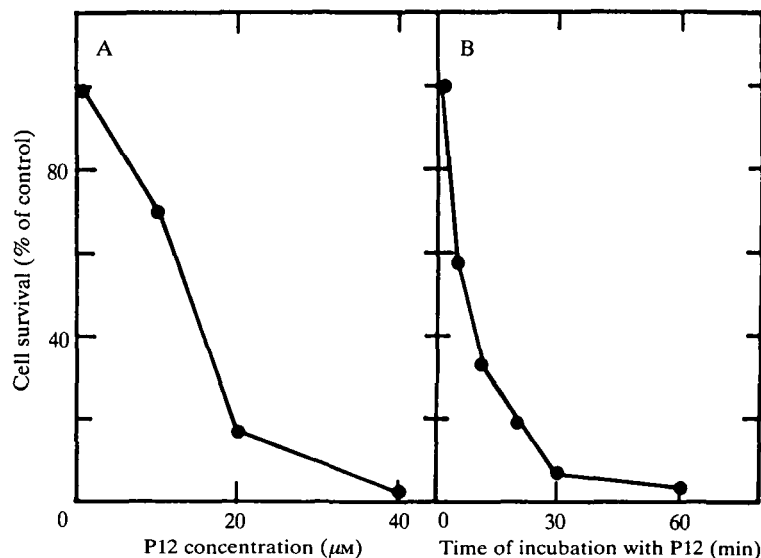


Fig. 3. Relation of cell survival to P12 concentration and time of incubation. A. HL-60 cells were incubated overnight in serum-containing medium with various concentrations of P12, washed, and irradiated at 366 nm for 2 min. B. HL-60 cells were incubated for various times with 20 μM -P12 in serum-containing medium, washed, and irradiated for 4 min. Cell viability in both experiments was determined 24 h following irradiation.

cells were incubated with P12 in the presence of increasing concentrations of FCS. Considerably greater resistance to u.v. killing was observed in these cells, relative to those grown in the absence of serum.

Addition of competing natural fatty acid also affected photosensitization induced by P12. When cells were incubated with 20 μM -P12 and 100 μM -palmitic acid in the absence of serum, toxicity was almost completely abolished. When bovine serum albumin was added to the incubation mixture, palmitic acid did not inhibit photosensitization. These results suggest that the free palmitic acid is inhibiting the uptake of P12 rather than the acid-albumin complex.

Low temperature during incubation with P12 and the age of the culture also affect the extent of sensitization of HL-60 to irradiation (Fig. 5A and B, respectively). These conditions, which are associated with a decrease in the cellular uptake and incorporation of P12, resulted in less sensitization to irradiation.

The above data suggested that this procedure could be used for inducing selective killing of various cells types differing in their relative degrees of sensitivity to u.v. irradiation following incubation with P12. Fig. 6A and B demonstrate such differences between HL-60 and MEL cells. The smaller degree of sensitization of MEL cells to u.v. irradiation was related to their considerably smaller uptake of P12 relative to HL-60 cells (Nahas *et al.* 1986). This difference was subsequently used to eliminate the latter selectively in a mixed population of HL-60 and MEL cells. Fig. 6C shows that by adjusting the conditions of incubation with P12 and the duration of irradiation it was indeed possible to eliminate more than 80% of the

HL-60 cells while most of the MEL cells remained unharmed and proliferated normally.

Differences in uptake of P12 into differentiated, relative to undifferentiated, malignant cells in a mixed population could be used to eradicate the latter specifically. For this purpose, HL-60 cells were cultured for 4 days in the presence or absence of 1.25 % DMSO, which caused their conversion to mature granulocytes, as evident morphologically following staining with May-Grunwald and Giemsa and by the ability of more than 90 % of the cells to reduce nitroblue tetrazolium. Cells grown without DMSO did not change their nature as immature promyelocytes and less than 1 % stained positively with nitroblue tetrazolium. When cells from each respective culture were incubated with P12 and subsequently irradiated, uninduced HL-60 cells (Fig. 7A) were found to be significantly more sensitive to u.v. irradiation than their differentiated counterparts (Fig. 7B). When cells from these two respective

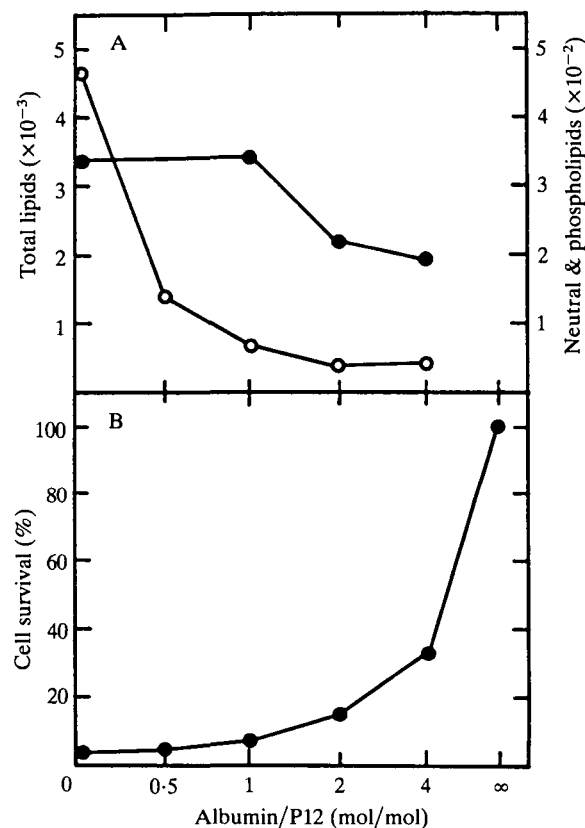


Fig. 4. The effect of albumin on P12 metabolism and photosensitization. HL-60 cells were washed and resuspended in serum-free medium, incubated for 30 min with $20 \mu\text{M}$ -P12 complexed to bovine serum albumin at various mole ratios, washed and irradiated for 2 min at 366 nm. A. Samples were removed for analysis of total (○) and esterified lipids (●). Results are expressed as fluorescence units. B. The remaining cells were incubated overnight at 37°C and their viability was determined.

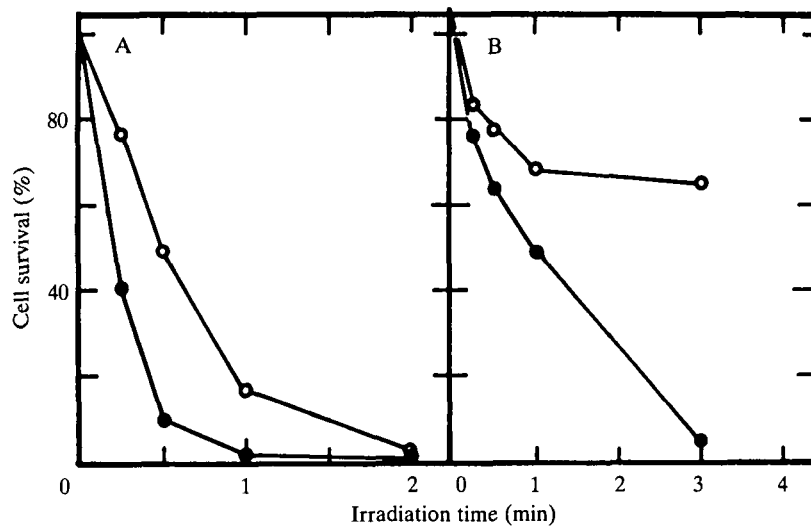


Fig. 5. The effect of age of the culture and temperature of incubation with P12 on photosensitization. A. Logarithmically growing HL-60 cells from 1-day-old culture (●) and cells from stationary, 7-day-old culture (○) were incubated with $20 \mu\text{M}$ -P12 for 10 min in serum-free medium. The cells were then washed and irradiated. B. Cells were incubated with P12 in serum-containing medium for 2 h at either 37°C (●) or 4°C (○). The cells were then washed and irradiated at either 4°C or 37°C , respectively. Cells were cultured overnight at 37°C and then the viability was determined.

cultures were mixed, incubated with P12 and irradiated, the differentiated cells survived selectively (Fig. 7C).

Similar results were obtained with MEL cells. When cultured in the presence of 4 mM-HMBA, MEL cells underwent maturation into haemoglobin-containing normoblasts, which could be distinguished from the uninduced cells by their characteristic morphology and by their ability to react with benzidine, indicating the presence of intracellular haemoglobin. More than 95% of the cells in the HMBA-treated cultures showed normoblast morphology and positive benzidine staining. Cells from both cultures were then incubated with P12 and subsequently irradiated; uninduced MEL cells (Fig. 7D) showed higher sensitivity to u.v. light than their differentiated counterparts (Fig. 7E). When a mixed population of these two respective types of cells was incubated with P12 and then irradiated, a selective survival of the differentiated cells was obtained (Fig. 7F).

DISCUSSION

Various compounds have been shown to exert a photosensitizing effect following their uptake by cells (Schothorst *et al.* 1977; Meager *et al.* 1982). In the present study a photosensitization procedure, based on the uptake of a pyrene-containing fatty acid that permits selective elimination of cells, is described. Incubation of cells with 12-(1-pyrene)-dodecanoic acid (P12), followed by irradiation with u.v. light at 366 nm resulted in cellular toxicity, which was determined by the Trypan Blue

exclusion test, and cloning efficiency in semi-solid medium. The toxicity was related to the intensity of irradiation and the rate and extent of the cellular uptake of P12. The latter could be manipulated by changing the incubation conditions, i.e. the P12 concentration, the presence of albumin, serum, competing non-fluorescent fatty acids, or the temperature. All those parameters also affected photosensitization. To

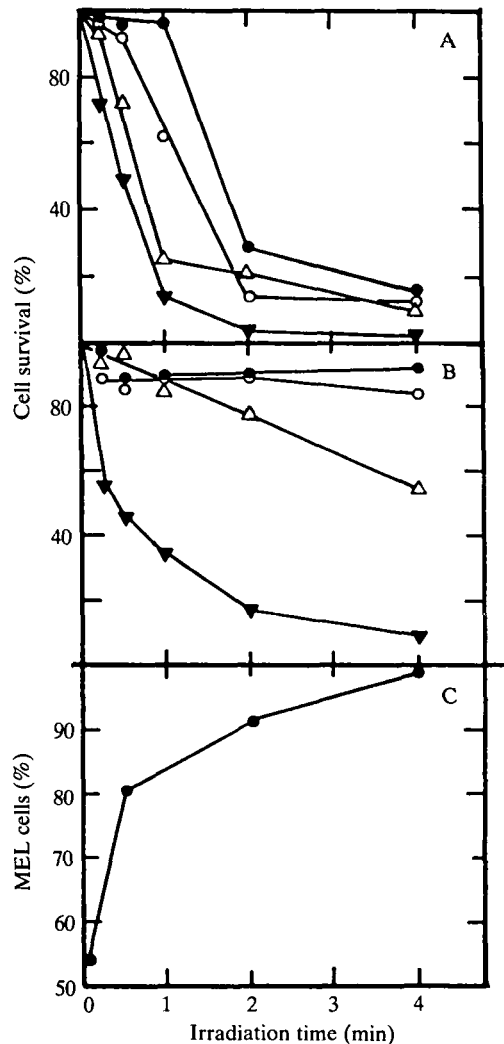


Fig. 6. Selective photosensitization and survival of MEL as compared to HL-60 cells. HL-60 (A) and MEL (B) cells were incubated in medium supplemented with $20 \mu\text{M}$ -P12 in serum-containing medium for 15 (●), 30 (○), 60 (△) or 120 min (▼). After washing, the cells were irradiated for the indicated times. Viability was determined 24 h following irradiation. C. HL-60 and MEL cells were mixed and then incubated with $20 \mu\text{M}$ -P12 in serum-containing medium for 30 min, washed, and irradiated; 24 h following irradiation cytopsin slides were prepared and stained with May-Grunwald and Giemsa. The percentage of MEL and HL-60 cells in each culture was determined. The results are presented as percentage of MEL cells in the culture.

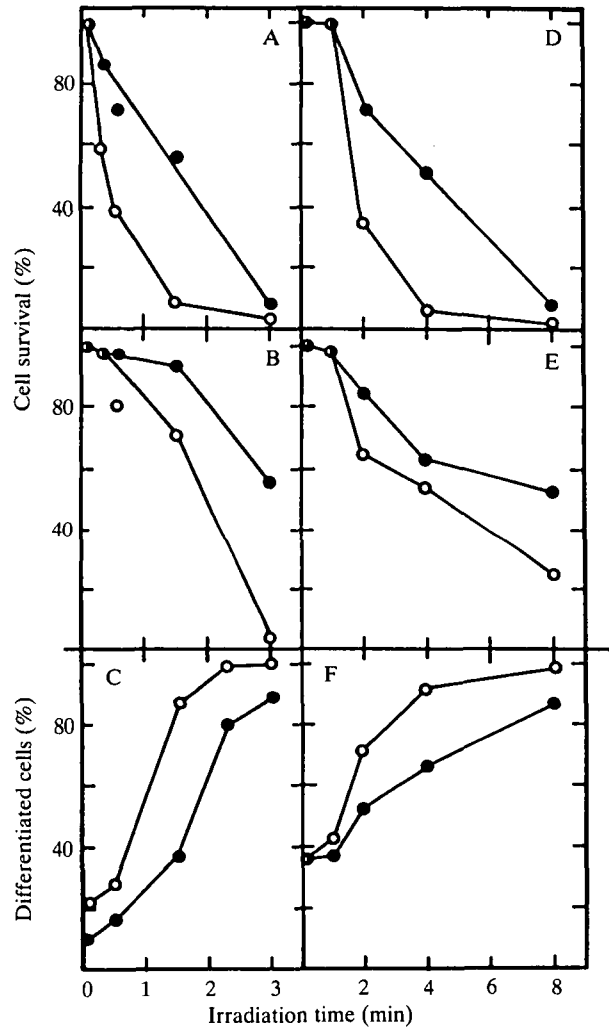


Fig. 7. Selective photosensitization and survival of differentiated relative to undifferentiated cells. HL-60 cells were cultured for 4 days in the absence (A) or presence (B) of 1.25% DMSO, washed and then incubated with 20 μM-P12 in serum-containing medium for 30 (●) or 90 min (○). Following washing the cells were irradiated, incubated overnight and cell viability was determined. C. HL-60 cells grown for 4 days in the absence or presence of 1.25% DMSO were washed, mixed and treated as in A. Following overnight incubation, cytopsin slides were prepared, stained with May-Grunwald and Giemsa, and the percentage of differentiated cells was determined morphologically. The results were confirmed by determining the percentage of nitroblue tetrazolium-reducing cells. MEL cells were cultured for 4 days in the absence (D) or presence (E) of 4 mM-HMBA, washed and incubated with 20 μM-P12 in serum-containing medium for 3 (●) or 6.5 h (○). Following washing, the cells were irradiated, incubated overnight and cell viability was determined. F. MEL cells were cultured for 4 days in the presence or absence of HMBA, mixed and then treated as in D and E. Following overnight incubation, the percentage of differentiated cells was determined by staining with benzidine.

determine the ability of this procedure to eliminate selectively one cell type and spare another more-resistant cell type, we used as a model system the HL-60 and MEL cells, since we have previously found that these cells differ in their uptake of P12 (Nahas *et al.* unpublished). The results indicated that HL-60 cells are considerably more sensitive than MEL cells and can be eliminated selectively from a mixture of both cell types. Our results also show that uninduced cells from both lines are more sensitive than cells induced to undergo differentiation. Preliminary results indicate that this procedure can be applied not only to artificially mixed populations, but also to naturally occurring cell mixtures such as blood and bone-marrow cells. The higher sensitivity of undifferentiated leukaemic cells, as compared to their differentiated non-leukaemic progeny, suggests that this procedure may be used in certain clinical situations for selective elimination of malignant cells (i.e. purging of bone marrow prior to autologous transplantation).

Previous studies, conducted exclusively with porphyrin derivatives, have shown that phototoxicity proceeds *via* the generation of singlet oxygen as a result of energy transfer to O₂ (Moan *et al.* 1979). Further studies are now in progress on the mechanism of the cytolytic effect exerted by pyrene-containing fatty acids and to investigate the possibility of applying the procedure for selective killing of certain subpopulations in naturally occurring cell mixtures.

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