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Induction, by adriamycin and mitomycin *C*, of modifications in lipid composition, size distribution, membrane fluidity and permeability of cultured RDM4 lymphoma cells

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Adriamycin and mitomycin C were previously found to modulate the sensitivity of lymphoma cells to lysis by certain effectors of immunity and this modulation was dependent on drug concentration. In the present studies, RDM4 lymphoma cells were treated with different concentrations of the two drugs for 24 h in culture. These treatments resulted in changes in the lipid composition, membrane fluidity, cell size distribution, and permeability to $^{51}CrO_4$, Trypan blue, Acridine orange and trimethylaminodiphenylhexatriene (TMA-DPH) of the cells. Changes in some of these parameters, as a function of drug concentration, resulted in dose-response curves which were bell-like shaped, hence paradoxical similarities between non-drug-treated cells and cells treated with higher drug concentrations were observed.

Introduction

Adriamycin interferes with cell growth not only at the level of nucleic acids, but also at the level of the outer membrane [1–3]. This latter action may modify cell membrane morphology [4] and/or physiological functions [5] as a function of drug concentration. Among the interactions between adriamycin and the cell surface, it is now a fairly well documented fact that suceptibility to the killing action of immune effectors can be modified. These modifications, however, are not univocal. For instance, treatment with adriamycin has been reported to increase susceptibility to lysis by lymphocytotoxins [6], macrophages [7], complement fixing antibodies [8,9], cytotoxic T lymphocytes and interleukin-2-induced LAK cells [10], but also to provide some degree of protection against killing by NK cells [11,12] or by complement fixing antibodies, depending on the conditions [13]. In fact, these modifications appear largely dependent on the drug concentrations used for the pretreatment of the prospective target cells [10,13]. The properties of adriamycin to modulate the sensitivity of target cells to destruction by immune effectors have often been compared to those of other anticancer drugs, such as mitomycin C, with some variations noted from one system to another [8–10,13].

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It has been proposed by Schlager that while both adriamycin and mitomycin C modulate sensitivity to lysis by cytotoxic T lymphocyte (CTL) or by cytotoxic antibodies, each have different effects on the physiochemical parameters of the cell membrane, in particular on the lipid composition [9], resulting in consistently different patterns of modulation [8]. In our system, we have observed more similar patterns of modulation of cell susceptibility to killing by immune effectors, exerted by adriamycin and mitomycin C treatments [10,13]. Since fatty acid composition and membrane fluidity have been shown to be involved in the susceptibility of some tumor cells to the cytotoxic effects of drugs and effector cells [8,9,14–22], we have examined the association of these two parameters with the viability of RDM4 lymphoma cells treated with different concentrations of adriamycin or mitomycin C and we report here our results.

Materials and Methods

(a) Cells and cell treatments

RDM4 lymphoma cells, derived from AKR mice, were routinely propagated in culture under standard conditions in 'complete RPMI-1640' medium as defined in Ref. 10. Cells were harvested 1 day after passage and treated with mitomycin C or adriamycin. In a few cases, as mentioned, cells were harvested after 3 days of culture. Harvesting of '1 day' and '3 day' cultured cells on the same day was performed as described [23]. The harvested RDM4 cells were washed twice in Earle's Balanced Salt Solution (EBSS), adjusted to $0.75 \cdot 10^6$ cells/ml in 'complete RPMI 1640' and incubated for 20 h, at 37 °C in a humid atmosphere with 5% CO_2, in the presence of the drug at different concentrations.

The incubation volumes varied from 0.2 to 4 ml (in either Linbro 96-well or 6-well tissue culture plates) to 10, 50 and 100 ml (in Linbro culture flasks of 25 cm^2, 75 cm^2 and 175 cm^2, respectively) according to the experiment. The cells were washed three times in EBSS before further manipulation. The drugs used were adriamycin (doxorubicin hydrochloride) and mitomycin C which were purchased from Sigma, St. Louis, MO.

(b) Analysis of fatty acid composition of the cells

Large quantities of drug-treated cells were washed five times in EBSS, and spun in 1.5 ml plastic Eppendorf tubes. The supernatant fluids were discarded, the tubes were saturated with nitrogen, sealed and stored at −70 °C until fatty acid analysis. Each experimental group consisted of quadruplicates 15–20 million cells each. Lipids were extracted from the cell pellets which had been resuspended in a 0.1 volume of phosphate-buffered saline with chloroform/methanol (2 : 1, v/v) containing 0.2% butylated hydroxytoluene in 16 × 125 mm tubes. After centrifugation, the lower phase was taken to dryness and redissolved in 100 µl of HPLC grade chloroform. Lipid classes were fractionated on Merck Silica thin-layer chromatographic plates with a solvent system consisting of hexane/diethyl ether/acetic acid (80 : 20 : 1, v/v). The total phospholipid fraction remaining at the origin was recovered for fatty acid analysis. The fatty acids of the phospholipid fractions were converted into methyl esters by treating them with 3 ml of boron trifluoride in methanol 14% (w/v), in screw-cap tubes under nitrogen for 30 min at 90 °C. After transesterification was completed, 2 ml of 0.09% saline was added, and the methyl esters were extracted with 5 ml of hexane. After centrifugation, the hexane phase was evaporated under nitrogen for immediate injection in the gas chromatograph or stored at −80 °C. Gas chromatography of the fatty acid methyl esters was performed in a Hewlett Packard Model 5880 A gas chromatograph equipped with a flame ionization detector, with a 6 foot long column of 2 mm i.d. packed with 10% Silar C on 100/120 Chrom Q11 (Mandell Scientific). The column was operated at 145 °C initially, increasing to 185 °C at a rate of 2 Cdeg/min with a gas flow of 30 ml/min of helium through the column. For quantitative analysis, the samples were injected with a known quantity of 17 : 0 methyl ester as the standard. Identification of the peaks was made by comparison of the retention times with those of the standards.

(c) Cell membrane fluidity determinations

The plasma membrane fluidity of differently treated cells was determined by measurement of fluorescence polarization of an hydrophobic fluo-

rescent probe, trimethylaminodiphenylhexatriene (TMA-DPH). This probe was chosen because of its specific localization to the plasma membrane [24,25] which was verified by fluorescence microscopy for the RDM4 cells. After three washings in Krebs-Ringer buffer, the cells were suspended at a concentration of 10^6 cells/ml. TMA-DPH, solubilized in dimethylformamide, was added to the cell suspension at a final concentration of $1 \cdot 10^{-6}$ M and incubated at 37°C for 5 min, the required time to obtain a stationnary fluorescence equilibrium. Fluorescence intensities were measured with an Aminco SPF 500 spectrofluorimeter (excitation beam: 360 nm; emission beam: 425 nm). The fluorescence polarization ratio was determined by the equation

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

with $I_{\parallel}$ and $I_{\perp}$ the emitted light components polarized parallely and vertically, respectively, to the polarization orientation of the excitation beam.

(d) Comparison of cell size distributions

The cells were resuspended in EBSS, ($5 \cdot 10^3$ cells/ml) and volumes of 0.5 ml were determined in a Coulter Counter, model 3502ZB1 (Coulter Electronics, Hialeah, FL) equipped with a 'channelizer' and a '*X-Y* Recorder II' (Coulter Electronics). The histograms of cell frequencies were recorded as a function of channel number, the larger cells distributing to the higher channels.

(e) Cell viability determinations

Three different criteria for the evaluation of cell viability were used.

(i) 'Spontaneous ^{51}Cr-release'. The cells were incubated for 1 h with $Na_2\,^{51}CrO4$ (Merck-Frosst, Pointe-Claire, Quebec) at 37°C in EBSS (100 μCi/ml; $4 \cdot 10^6$ cells/ml). After four washings, they were treated with one of the drugs (DT) in 96-well culture plates. Some of the cells were not drug treated (NT); whereas, some were treated with 0.3% (v/v) of sodium dodecylsulfate to ensure total cell death and maximum ^{51}Cr release (MAX) into the culture medium. The cells were centrifuged and the radioactivity of the supernatants was determined using a gamma counter. The 'spontaneous ^{51}Cr release' of DT-cells was compared to that of NT-cells by the formula

$$\frac{\text{cpm of DT cells} - \text{cpm of NT cells}}{\text{cpm of MAX} - \text{cpm of NT cells}} \times 100$$

(ii) Trypan-blue exclusion test. Trypan blue dye was added to drug-treated and control (NT) cells after washing. The results were expressed as percentage of dye positive (non viable) cells.

(iii) Acridine orange test. Drug-treated and control (NT) cells were washed and then dyed with Acridine orange (Sigma; 1 μg/ml) plus propidium iodide (Sigma; final concentration 12 μg/ml), washed and mounted for examination with a fluorescence microscope [26]. The percentage of dead cells was calculated as

$$\frac{\text{number of red cells}}{\text{total number of (red + green) cells}} \times 100$$

(f) Determination of the capacity of DNA synthesis

After various treatments, the cells were washed and put into the wells of a microtiter plate (Linbro) at $1 \cdot 10^5$ cells per well in a volume of 100 μl of 'complete RPMI 1640 medium'. They were incubated for 18 h under standard culture conditions. 1 μCi of ^{3}H-dThd (New England Nuclear Canada, Lachine, Quebec) was added 6 h before the end of the culture period. The cells were then harvested on Skatron® paper, using a multiple automated cell harvester (Flow Labs, Mississauga, Ontario). Radioactivity was determined by liquid scintillation. Each experiment was done in triplicate.

Results

(a) Cell fatty acid composition as a function of drug treatment

The results of representative experiments, shown in Table I and Table II, indicate that the relative composition of saturated and monounsaturated fatty acids of the cell phospholipids decreased as a function of increasing concentrations of adriamycin or mitomycin C. The monounsaturated fatty acids were more affected than the saturated fatty acids. Conversely, increasing the concentrations of both antimitotics resulted in a

TABLE I

FATTY ACID COMPOSITION (mg %) OF TOTAL PHOSPHOLIPID OF RDM4 LYMPHOMA CELLS CHALLENGED WITH ADRIAMYCIN

Fatty acid [a]	Adriamycin (μg/ml) [b]			
	0	0.1	2	12
16:0	21.2±0.2	20.2±0.4 *	19.0±1.6 **	18.9±0.5 **
16:1 (n−7)	4.4±0.2	3.8±0.1 **	2.8±0.3 **	3.0±0.3 **
18:0	11.0±0.5	9.7±0.1 **	11.6±0.3	12.1±0.1 *
18:1 (n−9)	29.5±0.6	28.1±0.3 *	23.5±0.5 **	23.6±0.1 **
18:2 (n−6)	3.5±0.8	3.9±0.1	3.9±0.2	3.9±0.3
18:3 (n−3)	2.6±0.1	2.6±0.1	1.9±0.1 **	2.0±0.0 **
20:3 (n−6)	2.4±0.1	2.9±0.1 **	3.5±0.2 **	3.1±0.0 **
20:4 (n−6)	9.3±0.1	11.8±0.2 **	15.9±0.6 **	14.4±0.3 **
20:5 (n−3)	0.5±0.1	0.6±0.0 *	0.8±0.0 **	0.6±0.0 *
22:4 (n−6)	1.5±0.1	2.1±0.2	2.5±0.2 **	2.9±0.1 **
22:5 (n−6)	1.1±0.2	0.6±0.3	0.5±0.0 **	0.5±0.0 **
22:5 (n−3)	4.1±0.1	4.8±0.1 **	5.5±0.3 *	5.9±0.1 **
22:6 (n−3)	4.3±0.1	4.7±0.1 **	5.1±0.3 *	5.8±0.1 **
Others	1.6±0.4	1.2±0.4	0.8±0.0	0.7±0.1
Total saturated	34.6±0.4	32.0±0.2 **	32.2±1.8	32.7±0.4 **
Total monounsaturated	33.9±0.7	31.8±0.3 **	26.3±0.2 **	26.6±0.2 **
Total polyunsaturated	29.8±0.5	34.8±0.2 **	39.9±2.0 **	39.4±0.6 **

[a] Fatty acids are abbreviated in the usual manner: the first number indicates the number of carbon atoms and is followed by the number of double bonds; the number after $n-$ indicates the number of carbon atoms between the methyl end of the molecule and the first double bond on this side.

[b] Mean ± S.D. of four cultures.

* $P < 0.05$; ** $P < 0.01$.

TABLE II

FATTY ACID COMPOSITION (mg %) OF TOTAL PHOSPHOLIPID OF RDM4 LYMPHOMA CELLS CHALLENGED WITH MITOMYCIN C

Fatty acid	Mitomycin C (μg/ml) [a]			
	0	1.5	12.5	50
16:0	21.2±0.2	20.5±0.0 **	19.6±0.5 **	17.6±0.9 *
16:1 (n−7)	4.4±0.2	4.1±0.1 *	3.4±0.1 **	2.8±0.2 **
18:0	11.0±0.5	9.9±0.1 *	10.4±0.5	12.3±0.6 *
18:1 (n−9)	29.5±0.6	28.6±0.4	25.4±0.7 **	23.4±0.2 **
18:2 (n−6)	3.5±0.8	3.9±0.5	4.0±0.4	3.9±0.2
18:3 (n−3)	2.6±0.1	2.4±0.1 *	1.9±0.2 *	1.7±0.1 **
20:3 (n−6)	2.4±0.1	2.6±0.1 *	3.1±0.1 **	3.2±0.1 **
20:4 (n−6)	9.3±0.1	11.4±0.3 **	14.2±0.4 **	14.9±0.3 **
20:5 (n−3)	0.5±0.1	0.6±0.1	0.7±0.0 **	0.6±0.0 *
22:4 (n−6)	1.5±0.1	1.7±0.1	1.8±0.1 *	2.5±0.2 **
22:5 (n−6)	1.1±0.2	0.8±0.1	1.1±0.3	1.0±0.8
22:5 (n−3)	4.1±0.1	4.6±0.1 **	5.4±0.2 *	5.9±0.1 **
22:6 (n−3)	4.3±0.1	4.6±0.1 *	5.2±0.3 *	5.9±0.0 **
Others	1.6±0.4	1.4±0.4	1.6±0.3	1.4±0.7
Total saturated	34.6±0.4	32.7±0.1 **	31.8±0.4 **	31.6±1.6 **
Total monounsaturated	33.9±0.7	32.7±0.4 *	28.8±0.6 **	26.2±0.1 **
Total polyunsaturated	29.8±0.5	33.5±0.9 **	38.3±0.4 **	40.4±1.1 **

[a] Mean ± S.D. of four cultures.

* $P < 0.05$; ** $P < 0.01$.

TABLE III

FATTY ACID AMOUNTS ($\mu g/10^6$ cells) IN TOTAL PHOSPHOLIPIDS OF RDM4 LYMPHOMA CELLS CHALLENGED WITH ADRIAMYCIN

Fatty acid	Adriamycin (μg/ml) [a]			
	0	0.1	2	12
16:0	41.7 ± 12.4	40.4 ± 0.4	25.5 ± 6.3 [c]	36.9 ± 18.9
16:1 ($n-7$)	6.7 ± 1.7	6.5 ± 0.3	5.2 ± 2.3	6.2 ± 3.8
18:0	18.9 ± 4.8	19.2 ± 2.9	16.3 ± 4.6	31.1 ± 12.9
18:1 ($n-9$)	47.9 ± 11.8	45.2 ± 0.7	27.1 ± 9.4 [b,c]	54.2 ± 22.3
18:2 ($n-6$)	7.3 ± 2.2	6.2 ± 1.4	5.5 ± 1.2 [c]	8.3 ± 1.7
18:3 ($n-3$)	2.7 ± 0.6	2.4 ± 0.3	1.6 ± 0.6 [b]	4.4 ± 2.6
20:3 ($n-6$)	4.9 ± 1.4	5.7 ± 0.4	3.4 ± 1.4 [c]	5.8 ± 1.3
20:4 ($n-6$)	25.8 ± 7.5	31.9 ± 1.1	19.1 ± 6.6 [c]	36.5 ± 8.7
20:5 ($n-3$)	0.7 ± 0.3	0.6 ± 0.1	0.4 ± 0.1 [c]	0.4 ± 0.3
22:4 ($n-6$)	4.0 ± 1.3	3.3 ± 0.1	2.0 ± 0.5 [b,d,e]	5.5 ± 2.1
22:5 ($n-6$)	0.8 ± 0.4	0.8 ± 0.3	0.7 ± 0.4	0.9 ± 0.9
22:5 ($n-3$)	11.7 ± 3.4	12.0 ± 0.5	6.8 ± 2.5 [c,f]	13.9 ± 2.9
22:6 ($n-3$)	10.4 ± 3.0	9.1 ± 0.3	5.9 ± 2.0 [e]	16.7 ± 6.7
Total saturated [g]	64.4 ± 11.0	63.0 ± 2.8	43.7 ± 10.7 [c]	70.2 ± 33.4
Total monounsaturated [g]	54.6 ± 13.4	51.7 ± 0.7	32.2 ± 9.2 [b,c]	60.4 ± 25.5
Total polyunsaturated [g]	69.3 ± 19.7	72.9 ± 3.1	46.0 ± 14.3 [c,e]	93.7 ± 26.7

[a] Mean ± S.D. of four cultures.
[b] $P < 0.05$ relative to no adriamycin treatment.
[c] $P < 0.05$ relative to treatment with adriamycin, 0.1 μg/ml.
[d] $P < 0.01$ relative to treatment with adriamycin, 0.1 μg/ml.
[e] $P < 0.05$ relative to treatment with adriamycin, 12 μg/ml.
[f] $P < 0.01$ relative to treatment with adriamycin, 12 μg/ml.
[g] Includes fatty acids not shown in the Table.

continuously increasing proportion of polyunsaturated fatty acids (PUFA). The comparison of individual fatty acids showed that 18:1 ($n-9$) accounted for the greatest change among the monounsaturated fatty acids although 16:1 ($n-7$) decreased more than 18:1 ($n-9$). The relative increase of the ($n-6$) fatty acids was more than twice that of the ($n-3$) fatty acids. Among the ($n-6$) fatty acids, the proportion of 22:4 increased the most, followed by 20:4 and 20:3. Among the ($n-3$) fatty acids, the proportion of 22:5 and 22:6 were equally increased. Table III demonstrates that the actual amounts of PUFA varied in a biphasic manner as a function of ADM concentrations. Except for four fatty acids, 16:1 ($n-7$), 18:0, 18:2 ($n-6$) and 22:5 ($n-6$), there was a general significant decrease in the absolute amounts of the fatty acids from cells treated with 2 μg/ml of adriamycin compared to cells treated with the other concentrations of adriamycin. However, the proportion of certain polyunsaturated fatty acids relative to 16:0 increased regularly with increasing concentrations of adriamycin, the proportion of most of the very long chain polyunsaturated fatty acids increasing only in cells treated with 12 μg/ml of adriamycin.

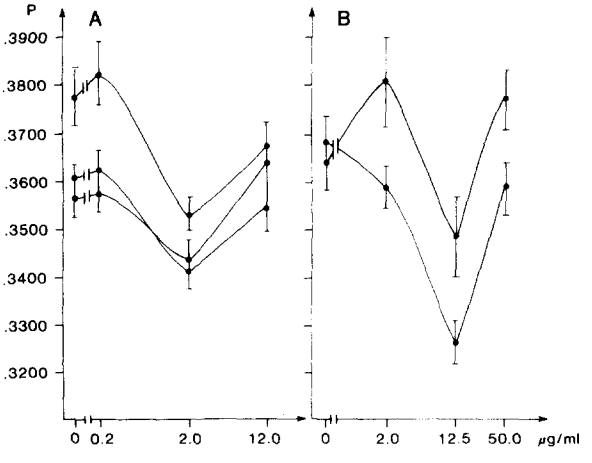

Fig. 1. Cell membrane rigidity, expressed as polarization P of TMA-DPH, as a function of drug concentration: (A) Treatment with adriamycin; (B) Treatment with mitomycin C. Vertical bars denote standard deviations. The results of three (A) or two (B) independent experiments are shown.

(b) Cell membrane fluidity, as determined by polarization of TMA-DPH

As seen in Fig. 1, the lower drug concentrations (i.e. 0.1 μg/ml of adriamycin, or 1.5 μg/ml of mitomycin C) might slightly, but insignificantly, increase the polarisation *P*. In contrast, mid-range drug concentrations (2 μg/ml of adriamycin, 12.5 μg/ml of mitomycin C) always decreased the *P* values significantly, denoting a dramatic increase in fluidity. Treatment with the higher drug concentrations (12 μg/ml of adriamycin, 50 μg/ml of mitomycin C) restored the high *P* values.

Photographs show that the fluorescence emitted by TMA-DPH was localized to the outer cell membrane of untreated normal cells (Fig. 2a). Figs. 2b and 2c show killed RDM4 cells or cell debris, which result from treatment with mitomycin C at the concentrations of 2 and 12.5 μg/ml. A widespread and intense fluorescence is seen, indicating penetration of the probe into the cytoplasm, as a result of drug-induced cell damage. On the other hand, in cells treated with 50 μg/ml of mitomycin C, the TMA-DPH distribution on the cell membrane seems to be essentially similar to that of control cells (Fig. 2d). The photographs also suggest an action of drug concentration on cell size.

In another experiment, RDM4 cells, cultured for 1 day, were compared to 3 day cultured cells. In this latter case, drug-induced relative changes in the *P* value, as well as in the percentage of Trypan-blue positive cells, were much less than in the case of the 1 day cultured cells (Table IV).

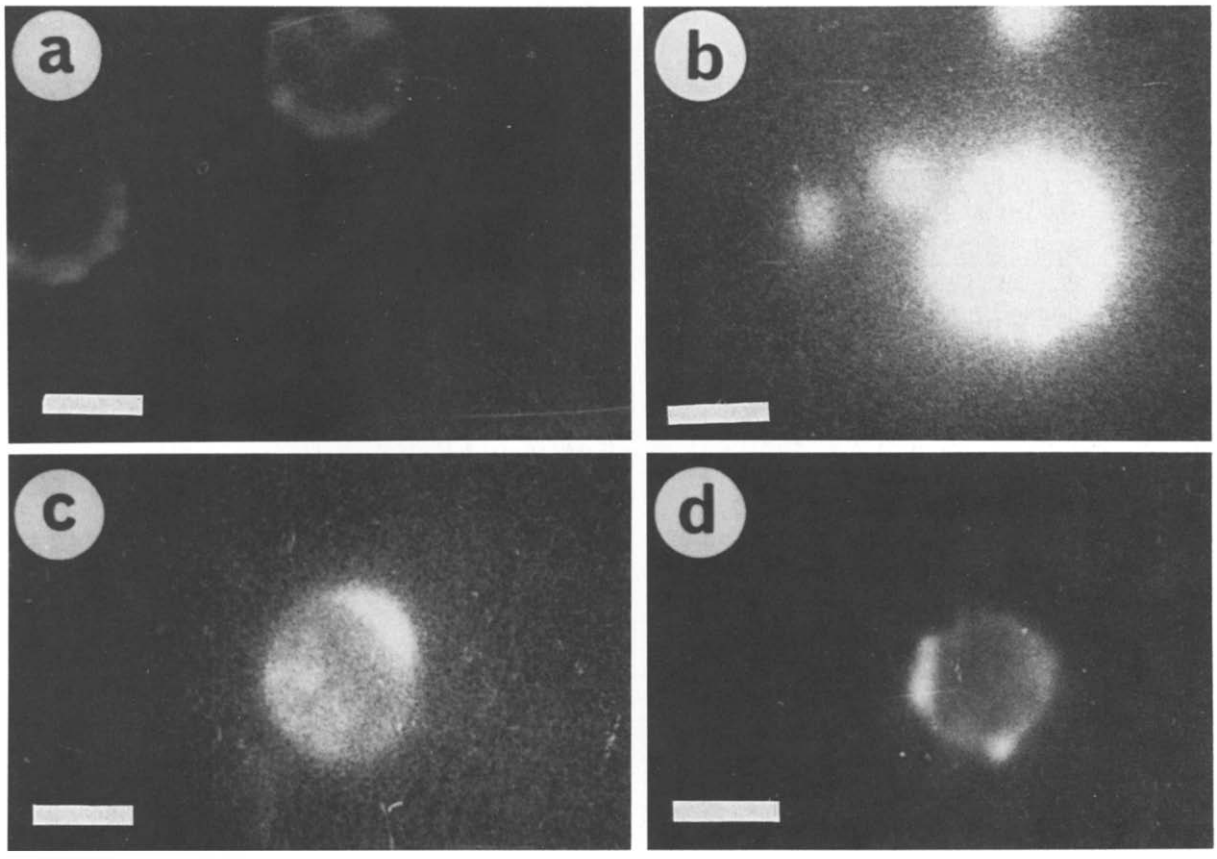

Fig. 2. Photographs of TMA-DPH-labelled cells after: no drug treatment (a), treatments with 12.5 μg/ml (b), 2 μg/ml (c) or 50 μg/ml (d) of mitomycin C. Bar = 5 μm.

TABLE IV

INFLUENCE OF CULTURE 'AGE' OF RDM4 CELLS ON MITOMYCIN C- OR ADRIAMYCIN-INDUCED CHANGES IN FLUIDITY (AS APPRAISED BY TMA-DPH POLARIZATION) AND ON PERCENTAGE OF TRYPAN-BLUE POSITIVE CELLS

MitC, mitomycin C; ADM, adriamycin.

Expt.	Treatment	Days of cultivation			
		1		3	
		P	% cell stained	*P*	% cell stained
1	none	0.3823	6%	0.3835	0%
	Mit C, 12.5 μg/ml [a]	0.3576	30%	0.3704	13%
2	none	0.3815	0%	0.3880	0%
	ADM, 2.0 μg/ml [a]	0.3493	16%	0.3683	11%

[a] Dose that induced the maximal membrane fluidification (see Fig. 1).

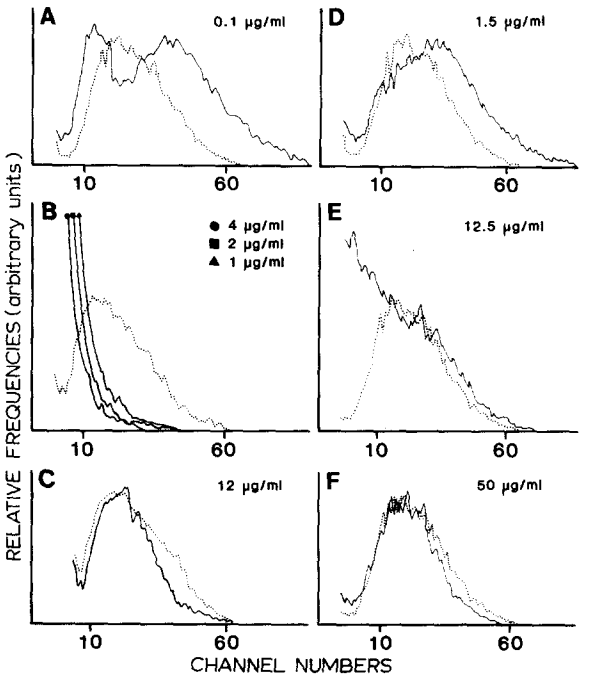

Fig. 3. Distribution of cells ('relative frequency') as a function of their size ('channel number'). Dotted line: non-drug-treated control cells; continuous line: drug-treated cells, at the concentration indicated. (A, B, C) Treatment with adriamycin; (D, E, F) Treatment with mitomycin C.

(c) Influence of drug-concentration on the size distribution of drug-treated cells

As shown in the upper panels of Fig. 3, treatment with 0.1 μg/ml of adriamycin (A) or 1.5 μg/ml of mitomycin C (B) tended to elicit a cell population of larger cell size than in the case of untreated cells. In contrast, treatment with 1 to 4 μg/ml of adriamycin (B), or 12.5 μg/ml of mitomycin C (E), produced a large number of particles of reduced size, probably cell debris. Increasing the concentration of adriamycin to 12 μg/ml (C) or of mitomycin C to 50 μg/ml (F), gave a surprisingly 'normal' cell size distribution, similar to that of the controls. These experiments have been repeated several times.

(d) Estimation of cell membrane permeability according to three classical criteria

RDM4 cells, treated with different concentrations of adriamycin or mitomycin C, were evaluated as the percent of cells sensitive to the drug treatments (Fig. 4).

Augmenting the drug concentrations resulted in an increase in percentage of Trypan blue and Acridine-orange positive cells observed on the one

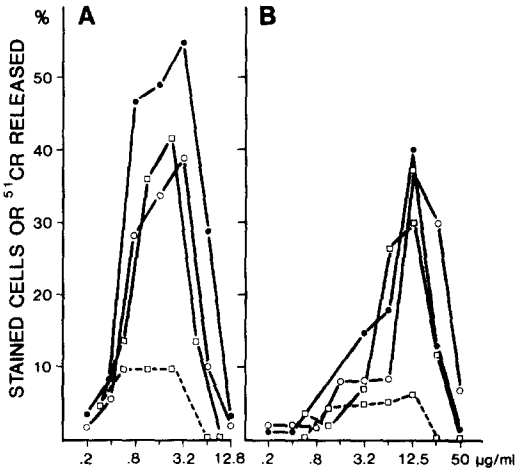

Fig. 4. Estimation of cell membrane permeability, as a function of drug concentration. Dotted line: 3 day cultured cells. Continuous line: 1 day cultured cells. (A) Treatment with adriamycin. (B) Treatment with mitomycin C. Results are expressed as 'spontaneous ^{51}Cr release' calculated as indicated in Materials and Methods (□), as percentage of Trypan blue stained cells (○) or as percentage of red cells in the Acridine orange + propidium iodine-treated cells (●).

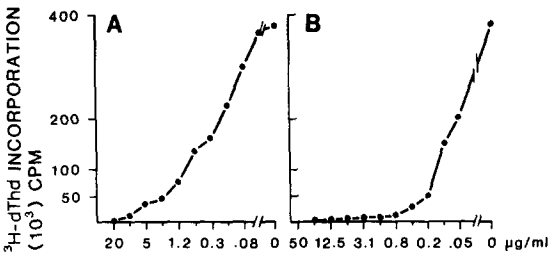

Fig. 5. DNA synthesis, as measured by ^{3}H-dThd uptake, as a function of drug concentration. The means of triplicated experimental points are expressed in cpm. (A) Treatment with adriamycin. (B) Treatment with mitomycin C.

hand, or of spontaneous ^{51}Cr release on the other hand. However, surprisingly, further increases resulted in a diminution in the percentage of cells which would be classified as 'dead' according to spontaneous ^{51}Cr release, or to dye penetration. The three criteria gave remarkably concordant results. The observed bell-shaped dose-response curves were sharp when the drug treatments were applied to 1 day cultured RDM4 cells. They were still observed, but considerably attenuated, when the treated cells had been cultured 3 days before applying the drug treatments.

Using another criterion, DNA synthesis capacity appraised by ^{3}H-dThd uptake, led to the more expected pattern of a regular decrease in RDM4 cell biochemical activity, as drug concentrations increased (Fig. 5).

Discussion

The experiments described in this article were designed to compare certain biochemical and biophysical parameters of populations of RDM4 lymphoma cells treated with different concentrations of either adriamycin or mitomycin C. The parameters used were chosen in the hope of clarifying their link with the ability of these antimitotics to modify the susceptibility of RDM4 cells to cytolytic immune effectors.

The fatty acid composition of drug treated cells exhibited a continuous shift towards less monounsaturated fatty acids and more polyunsaturated fatty acids (PUFA), as drug concentrations increased. However, cells treated with 2 µg/ml of adriamycin contained lesser amounts of a variety of fatty acids than the cells treated with 0.1 or 12 µg/ml of adriamycin. This decrease seemed to parallel the diminution in size of the cell treated with 2 µg/ml of adriamycin. The reason for the diminution in cell size at that particular concentration of adriamycin is presently unknown. Our results are consistent with those of Vrignaud et al. [22], who demonstrated that the in vitro differentiation of glial cells and the acquisition by the same cells of a resistance to adriamycin was accompanied by an increase in the PUFA proportion, to the detriment of the monounsaturated series. Tumor cells have decreased essential fatty acid (EFA) desaturase activities [27] and have a low PUFA content [28]. The acyl group composition of cells in culture is known to be dependent on the composition of the serum in the culture medium [29]. Accordingly, it is not known at the present time if the differences observed are the result of differences in cell uptake of the EFA present in the culture medium, or of differences in the biotransformations of these EFA to longer chain PUFA.

The toxicity of adriamycin was first considered to be directed mainly against the DNA, but is now thought to be due in part to lipid peroxidation in the membrane [30]. However, this mechanism of anti-cancer action has been challenged recently in favor of a mechanism involving oxygen radicals [30–32]. Although the cell surface membrane has been shown to play a major role in the cytotoxic action of adriamycin [33,34], is not clear whether the drug-induced radicals act directly on sensitive cellular constituants, unrelated to the content of peroxidable PUFA, or exert their effects through a secondary event such as lipid peroxidation. The toxicity of oxygen, or of its radical derivatives, is often accompanied by the peroxidation of unsaturated fatty acids. A vital question with respect to this observation is whether lipid peroxidation represents a coincidental outcome of radical-mediated damage or if lipid peroxidation products are directly deleterious to the cells. In our system, it was evident that polyunsaturated fatty acids accumulated in the adriamycin-treated cells without being peroxidized, suggesting that the ability of adriamycin to kill RDM4 cells is independent of lipid peroxidation. Protection from peroxidation may be brought about by sequestering the PUFA in an inert metabolic pool or by increasing the

antioxydative potential of the cell. Recently, it was reported that the toxicity of oxygen radicals can be independent of the content of peroxidizable PUFA [35,36].

These results also suggest that the PUFA accumulating in the cells are protected from peroxidation. Since adequate provision of unprotected PUFAs can kill tumor cells selectively [37,38] and cell-mediated tumoricidal activity may depend in part on the accumulation and availability of certain PUFA [16,39,40], the accumulation of PUFA in RDM4 cells following treatment with adriamycin may enhance their susceptibility to cytolytic cells, if one hypothesizes that the release of the protection from peroxidation depends in part on the proteolytic activity of some enzymes or on some other unknown function provided by the cytolytic cells.

Fluorescence polarization of TMA-DPH, which was found to reflect events localized to the external membranes of living cells (Refs. 24, 25 and Fig. 2a), exhibited significant decreases in P values, as drug concentrations augmented. However, as drug concentrations reached 12 μg/ml of adriamycin or 50 μg/ml of mitomycin C, apparent rigidification of the cell membrane was observed. On the other hand, a possible interaction between the drug and TMA-DPH, with some consequences on P, cannot be excluded. This parameter was much less sensitive to drug action if tested on 'old', 3 day cultured cells instead of 'young', 1 day cultured RDM4 cells (Table IV). Thus, on the basis of polarization ratio value, the cells treated with mid-range concentrations (e.g. 1–4 μg/ml of adriamycin) reached a maximal fluidity, whereas cells treated with higher concentrations (e.g. 12 μg/ml of adriamycin) apparently behave like controls, not treated RDM4 cells. It is evident that the changes in acyl composition of the phospholipids did not contribute significantly to the differences in the fluidity. This important fluidification probably corresponds to membrane destabilization, which could be the origin of the increment of apparent cell lethality observed with these doses. When cell size distributions were considered, the same conclusion was reached (Fig. 3). In addition, it could be seen that treatment with the lower drug concentrations elicited the emergence of a population of larger cell size (Fig. 3A and 3D). The meaning of this change is not known, but it has already been reported that these low drug concentrations, in particular 0.1 μg/ml of adriamycin, are able to elicit significant changes in some functional properties of the membrane of RDM4 [13] or of other cells [5]. However, some kinds of drug-cell interactions are subject to important variations from one system to another [41].

Then, using either the dye penetration of cells or 'spontaneous' release of previously incorporated ^{51}Cr, frequently used criteria for mortality, we observed maximum values with some drug concentrations, with an apparent return to 'normal' values as the concentrations were further increased (Fig. 4). That cells treated with higher concentration seem to behave like non-treated cells was also visualized by fluorescence of TMA-DPH treated cells (Fig. 2). This pattern was no longer found, using a criterion such as capacity to synthesize DNA, where an expected regular dose-response effect was observed (Fig. 5). Cell penetration by dye would directly result from drug-induced augmentation of membrane permeability, and such an action has been reported in the case of adriamycin [42]. Membrane permeability to non-electrolytes has been reported to be dependent on fatty acid composition and to be proportional to fluidity [43]. The decreased permeability to dye penetration observed with the highest drug concentrations (Fig. 4) could therefore be related to the augmentation of the rigidification observed under the same experimental conditions (Fig. 1).

We therefore conclude that we could not find differential responses to adriamycin and to mitomycin C, at different concentrations of the drugs, with the criteria used. We observed bell-like dose-response curves both with adriamycin and with mitomycin C, using several criteria of cell viability after treatments with increasing drug concentrations.

Using another lymphoma of murine origin, the YAC lymphoma, we observed bell-shaped responses of ^{51}Cr release as a function of drug concentration with both adriamycin and mitomycin C treatments. However, the critical concentrations were not the same as with RDM4 [44]. With the P815 murine mastocytoma, a high cytotoxic action of adriamycin was reported with the con-

centration of 10^{-6} M (0.6 μg/ml), but no apparent decrease of toxicity was described with higher concentrations [45].

However, the fact that high drug concentrations may, at least in some cases, exhibit properties apparently similar to those obtained with non-treated cells must be underscored, to prevent the putting forth of misleading interpretations when assessing the viability of drug-treated cells by classical tests.

In addition to the presently reported effects of different drug concentrations, the existence of other possible major changes must be considered. It has recently been shown that a brief exposure time of tumor cells to 1 μg/ml of adriamycin was able to bring about qualitative as well as quantitative major chromosomal changes, in relation to the establishment of drug-resistant sublines [46]. These observations suggest that, with the conditions that we used, comparable major adaptative changes could also have occurred in the lymphoma cell population, and that the data we obtained on the cell membrane fluidity, cell size and fatty acid compositions could result both from direct drug actions and from drug-induced major biochemical alterations in the metabolism of the cell [46–48]. Without doubt, such phenomena must be studied in further experiments, to help understand the action of various drug treatments on the sensitivity of tumor cells to immune lysis.

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