J. Membrane Biol. 104, 107–118 (1988)

The Journal of
Membrane Biology

Correlation Between the Synergistic Effect of Liposomes and Endotoxins on the Activation of Macrophage Tumoricidal Activity and the Effect of Liposomes on the Rough Endoplasmic Reticulum of Macrophages

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Summary. Treatment of resident peritoneal macrophages of rats with small unilamellar vesicles of dipalmitoylphosphatidylcholine (DPPC SUV) potentiated their activation for tumor cell lysis by endotoxins. The fluorescence polarization of diphenylhexatriene (DPH) embedded in rough endoplasmic reticulum membranes isolated from DPPC SUV-treated macrophages was enhanced. The average fluorescence lifetime of DPH and the rotational correlation time deduced from anisotropy decay were unchanged, whereas the residual anisotropy and hence the order parameter were increased. The measurement of the fluorescence anisotropy of DPH as a function of the temperature showed a phase transition. No phase transition was observed in the rough endoplasmic reticulum membranes of macrophages either treated or not treated with cholesterol/DPPC SUV (1/1; mol/mol). The synergistic effect of DPPC SUV on the tumoricidal activity of macrophages induced by endotoxins appears to be correlated with the changes in the properties of the rough endoplasmic reticulum membranes. Both effects were transient; they had the same kinetics of induction and reversion, and they were both inhibited by cholesterol.

Key Words liposomes · endotoxins · macrophage cytotoxicity · rough endoplasmic reticulum · fluorescence polarization · fluorescence anisotropy decay

Introduction

For several years it has been hypothesized that macrophages may play a role against emerging or established tumors or metastases. To date this hypothesis remains controversial although macrophages may be activated for tumor cell killing, for example by muramyldipeptide encapsulated in liposomes in vivo (Fidler et al., 1982) and by interferon ex vivo (Stevenson, Foon & Sugarbaker, 1986). The general significance of these findings is at the origin of the controversy. There are doubts as to a) the extent of macrophage infiltration into tumors, b) the targeting of immunomodulators to intra- or peri-tumoral macrophages or the targeting of activated macrophages to tumor cells, c) the small fraction of macrophages responding to an immunomodulator and d) the short-lived tumoricidal activity of activated macrophages and their refractoriness to reactivation.

Understanding the mechanisms of macrophage activation for tumor cell killing and of the mechanisms by which activated macrophages kill tumor cells could improve immunotherapy protocols. Endotoxins are probably the most studied among the immunomodulators. Functional changes in macrophages activated by endotoxins for tumor cell lysis have been described but the cellular or molecular processes of activation are unknown. In vitro, endotoxins induce extracellular release by macrophages of arginase (Ryan, Yohe & Morrison, 1980) and/or cytotoxic factors (Hammerstrom, 1982; Kull & Cuatrecasas, 1984; Matthews, 1984; Sone, Lopez-Berestein & Fidler, 1985). Endotoxins also induce changes in the intracellular activity of lysosomal enzymes, such as β-glucuronidase (Morland & Morland, 1978), and in the metabolism of rRNA (Varesio, 1985).

In order to enhance macrophage-mediated cytolysis liposomes have been used in association with endotoxins for the purpose of modifying the membrane lipid composition of macrophages and/or tumor cells. In previous reports we evidenced a synergistic effect between dipalmitoylphosphatidylcholine small unilamellar vesicles (DPPC SUV)[1]

[1] *Abbreviations:* DPPC, dipalmitoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; RER, rough endoplasmic reticulum; SUV, small unilamellar vesicles.

and endotoxins on the activation of rat resident peritoneal macrophages for tumor cell lysis (Jeannin et al., 1983, 1985). In the present report modifications induced by liposomes at the level of the external or internal membranes isolated from macrophages or tumor cells were studied. These modifications were followed by measurement of the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH), the probe proposed by Shinitzky and Barenholz (1974) to specifically measure the microviscosity of the membranes. In fact, it became progressively clear that the steady-state polarization of DPH probed the structure order of the lipids rather than their true fluidity (Chen et al., 1977; Hare & Lussan, 1978; Sene et al., 1978; Lakowicz, Prendergast & Hogen, 1979). Therefore, we also measured the fluorescence anisotropy decay. Indeed, nanosecond time-dependent depolarization measurements allow insight into the rotational behavior of the probe and the lipid order within the membrane. Hence, more detailed information regarding both fluidity and structure changes within the microenvironment of the probe may be ascertained. Structural changes in the membranes were studied, moreover, by following the temperature profile of the fluorescence polarization in order to detect any possible phase transition.

Materials and Methods

Chemicals

Cholesterol (C) and dipalmitoylphosphatidylcholine (DPPC) were purchased from Sigma Chemical Company (St. Louis, Missouri). They were examined for purity by thin-layer chromatography on silicagel plates; a single spot was observed with 100 μg loading. Endotoxins (LPS W, *E. coli* 0128 B12) were purchased from Difco (Detroit, Michigan), and 1,6-diphenyl-1,3,5-hexatriene (DPH) from Fluka (Buchs, Switzerland).

Liposomes

The lipids used to prepare liposomes were dissolved in chloroform and a film of phospholipid (or lipid mixture) was obtained under reduced pressure by evaporating chloroform in a rotary evaporator for 1 hr at 45°C. The film of lipids that formed on the round flask wall was suspended by adding Ham F10 medium (Microbiological Associates, Walkersville, Maryland). The suspension was transferred into a tube and was vortex-mixed for 10 min to prepare multilamellar vesicles (MLV). The MLV preparation was sonicated under nitrogen for 1 hr at 50°C in a 20 kHz sonication bath, and centrifuged 1 hr at 30,000 × *g*, at 45°C to obtain small unilamellar vesicles (SUV). The supernatant alone was used after sterilization by filtration through 0.2 μm filters (Gelman, Ann Arbor, Michigan). The yield of DPPC SUV preparation was checked by gas chromatography using a Packard 428 chromatograph (Packard Instrument Co., Downers Groves, Illinois) fitted with an OV 351 capillary column (Spiral, Dijon, France). Palmitic acid was quantified as methylester using heptadecanoic acid as internal standard. The yield of DPPC SUV preparation determined through five experiments was 80 ± 5%. Liposome effect on cell viability was checked by a direct microscopy test with vital dye (neutral red or trypan blue, Merck, Darmstadt, Germany) and by a ^{51}Cr release assay.

Macrophages

Resident peritoneal macrophages were obtained by washing the peritoneal cavity of inbred BD IX rats with 10 ml Ham F10 medium containing 0.05 g heparin/liter (Liquémine, Roche, Neuilly, France). The macrophage content of peritoneal washing was evaluated by counting the cells adhering to a hemocytometer after a 30-min incubation at 37°C.

Tumor Cells

A permanent cell line, DHD K12/TS, established by trypsinization of dimethylhydrazine induced BD IX rat colon carcinoma (Martin et al., 1983) was used as target cells. Five to 10 × 10^6 tumor cells were incubated for 18 hr with 50 μCi ^{3}H-thymidine (25 Ci/mmol, CEA, Gif/Yvette, France) in 5 ml Ham F10 medium supplemented by 10% fetal calf serum (Hy-Clone, Sterile System Inc., Logan, Utah) and 40 μg gentamycin/ml. The latter is referred to as complete medium. Labeled tumor cells were used as target cells after detachment by treatment with 10 mg EDTA/ml, then 2.5 mg trypsin/ml in Ca^{2+} and Mg^{2+} free Hank's solution (Microbiological Associates).

Macrophage-Mediated Cytolysis Assay

Only the culture media and sera which did not contain endotoxins, as detected by the Limulus Amebocyte Lysate Test (Microbiological Associates), were used. Cell incubations were performed at 37°C. Macrophage-mediated cytolysis was assayed by ^{3}H-thymidine release as previously described (Jeannin et al., 1983). Briefly, peritoneal macrophages were plated in even wells of microtest 3040 tissue culture plates (Falcon, Oxnard, California) at a concentration of 10^5 macrophages/well. Labeled tumor cells were added to each well, 10^4 cells/well, with or without liposomes and/or endotoxins. The plates were incubated for 72 hr and, at the end of the incubation, were vigorously washed. The remaining viable adherent cells were lysed with sodium hydroxide and their radioactivity was counted. The macrophage-mediated cytolysis was calculated in percentage according to the formula $100 \times a-b/a$ where a is the mean residual radioactivity in the control wells and b is the mean residual radioactivity in the experimental wells containing macrophages. Experimental results were analyzed for their statistical significance by Student's t-test and Mann and Whitney's U-test.

Membrane Preparation

Macrophages or tumor cells were cultured on hydrophobic Teflon® in Petriperm dishes (Heraeus, Les Ulis, France); initial seeding was performed at 2.5 × 10^5 cells/cm^2. They were cultured at 37°C in complete medium with or without 2 mM DPPC SUV, 8 μmol DPPC/10^6 cells. At the end of the incubation time,

the culture media were discarded and the cultures washed with complete medium. The cells were harvested by detaching them with a rubber policeman and centrifuged 5 min at 150 × *g*. The pellet was washed 3 times with complete medium then suspended in 10 volumes (1 ml) of 1 mM $NaHCO_3$, 2 mM $CaCl_2$ and 5 mM $MgCl_2$ for 15 min at 4°C. The cells were homogenized in a Thomas homogenizer (size AA) with 12 strokes. The disrupted cells were then layered over a discontinuous saccharose gradient (4.75 ml of 70% saccharose, 9 ml of 42% saccharose and 9.25 ml of 20% saccharose in 2 mM $CaCl_2$ and 5 mM $MgCl_2$). The samples were centrifuged 1 hr in a Beckmann L8-55 ultracentrifuge using a 42-1 rotor at 52,000 × *g* and 4°C. Membranes recovered at the 42-20% saccharose interface were washed twice by centrifuging them at 13,000 × *g* and 4°C for 15 min first in 20% saccharose in 1 mM $NaHCO_3$ and secondly in 1 mM $NaHCO_3$. This method for the preparation of plasma membranes was taken from Koizumi et al. (1981).

Electron Microscopy

Membrane preparations were fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4 at 4°C for 1 hr). Samples were dehydrated in ascending concentrations of ethanol and embedded in glucidyl-ether (Cipec, Paris). Ultrathin sections were cut and stained with uranyl acetate and lead citrate and examined with a Hitachi HU-11 transmission electron microscope.

Steady-State Fluorescence Depolarization

A home-built spectrofluorimeter described earlier (Tatischeff & Klein, 1976; Klein, Ballin & Tatischeff, 1982) was transformed for the measurement of the steady-state fluorescence polarization. The excitation beam first depolarized by a quartz depolarizer of Lyot (Fichou, Fresne, France) was then split by a quartz beam splitter. One beam was directed onto a quantum counter (3 g/liter rhodamine B in methanol), the second onto the sample through a sheet polarizer (HNP'B polaroid) with its polarization vertically oriented. The fluorescence emission was analyzed at 90° through two polarizers (HNP'B Polaroid) oriented parallely and perpendicularly to the direction of polarization of the excitation beam. A stepping motor alternatively positioned each polarizer to measure the parallel (I_{VV}) and perpendicular (I_{VH}) components of the fluorescence emission. The ratio I_{VH}/I_{VV} measured for DPH 10^{-6} M in hexane at 20°C (excitation 350 nm, emission 430 nm) was used as correction factor (C) to determine the polarization of the probe in the membrane

$$p = (C \cdot I_{VV} - I_{VH})/(C \cdot I_{VV} + I_{VH}) \quad (1)$$

or its anisotropy

$$r = (C \cdot I_{VV} - I_{VH})/(C \cdot I_{VV} + 2I_{VH}). \quad (2)$$

The spectrofluorimeter was interfaced with a 9835A Hewlett-Packard microcomputer for entering, treating and storing the data.

The DPH probe stored at −20°C as a 2×10^{-3} M solution in tetrahydrofuranne was diluted to 8 μM in 17 mM phosphate buffer (pH 6.8) and vigorously dispersed by vortexing just before each experiment. In a 2 × 10 mm quartz cell were placed 0.45 ml of the membrane suspension ($A < 0.15$ at 430 nm) and 0.15 ml of the DPH diluted solution (2 μm final concentration). A second cell with 0.45 ml of the same membrane suspension and 0.15 ml of phosphate buffer (pH 6.8) was used to subtract the background of I_{VV} and I_{VH} components from the DPH signals.

Fluorescence excitation and emission spectra of the probe can be measured with the same apparatus.

Nanosecond Time-Dependent Fluorescence and Anisotropy Measurements

Time-resolved measurements were performed with the apparatus and methods earlier described (Gallais, Vincent & Alfsen, 1982). Fluorescence lifetimes were best described by a double exponential decay curve according to

$$I(t) = A_1 \cdot \exp(-t/T_1) + A_2 \cdot \exp(-t/T_2) \quad (3)$$

where $I(t)$ is the decay of the total fluorescence with time, T_1, T_2 and A_1, A_2 are the fluorescence lifetimes under double exponential approximation and their respective decay amplitudes. The mean excited state lifetime was

$$\langle T \rangle = (A_1 \cdot T_1^2 + A_2 \cdot T_2^2)/(A_1 \cdot T_1 + A_2 \cdot T_2). \quad (4)$$

The anisotropy decay was deconvoluted with a monoexponential function plus a constant term according to

$$r(t) = (r_0 - r_\infty) \cdot \exp(-t/\phi) + r_\infty \quad (5)$$

where $r(t)$ is the decay fluorescence anisotropy with time, r_0 is the anisotropy at time $t = 0$, r_∞ the residual anisotropy and ϕ the apparent rotational correlation time.

The steady-state anisotropy, r_s can be expressed by the sum of two terms deduced from the above relations

$$r_s = r_f + r_\infty \quad (6)$$

where r_f represents the dynamic concentration from the fast decaying components and r_∞ represents the static contribution from the residual component of $r(t)$ to the steady-state anisotropy (Hildenbrand & Nicolau, 1979).

The residual anisotropy, r_∞, present for times much longer than the rotational correlation time revealed that the motion of the DPH probe is hindered in the membranes. This orientational constraint may be characterized by the order parameter (Heyn, 1979)

$$S = (r_\infty/r_0)^{1/2}.$$

In the Kinosita model (Kinosita, Kawato & Ikegami, 1977) the DPH probe is assumed to wobble in a cone of half-angle θ_c, which can be deduced from the relation

$$S = \tfrac{1}{2} \cos \theta_c \cdot (1 + \cos \theta_c). \quad (7)$$

The cone angle characterizes the range of motion of the probe. The rotational correlation time, ϕ, characterizes the rate of motion of the probe. This rate depends on the viscosity of the medium in which the probe is assumed to wobble. A "viscosity in the cone," η_c, may be estimated from the relation

$$\eta_c = kT/6D_w V_e f \quad (8)$$

where k is the Boltzman constant and T, the absolute tempera-

Table 1. Reversibility of the DPPC SUV effect on the macrophage-mediated cytolysis induced by endotoxins[a]

Treatment and sequence duration					Residual dpm (× 10^2)		Cytolysis[b] (%)	DPPC SUV Effect[c]
0	18	30	//	72 hr	without RPM	with RPM		
——————!		——————!		——————	281 ± 11	318 ± 12	−13% (1)	—
——————!+++++++++!				——————	267 ± 17	223 ± 9	19% (2)	—
OOOOOOOO!+++++++++!				——————	284 ± 8	148 ± 8	48% (3)	$P < 0.01$
OOOOOOOO!		——————!		——————	275 ± 10	269 ± 10	2% NS	—
——————!		——————!		+++++++++++++++++	281 ± 11	244 ± 15	13% (1)	—
OOOOOOOO!		——————!		+++++++++++++++++	272 ± 9	259 ± 14	5% NS	NS
——————!OOOOOOOOO!				+++++++++++++++++	273 ± 10	200 ± 11	27% (3)	$P < 0.05$
——————!OOOOOOOOO!				——————	270 ± 12	269 ± 10	0% NS	—

[a] In vitro cytolysis mediated by resident peritoneal macrophages (RPM). RPM and prelabeled tumor cells were treated with (+++) or without 10 μg endotoxins/ml and with (ooo) or without 1 mM DPPC SUV for the indicated time and washed (!) then incubated in complete medium (—). Results are the mean value of eight cultures ± SD. These are representative data from three independent experiments.

[b] Statistical significance of RMP-mediated cytolysis (Student's *t*-test); NS: not significant; (1) $P < 0.05$; (2) $P < 0.01$; (3) $P < 0.001$.

[c] Statistical significance of the DPPC SUV effect on the induction by endotoxins of the RMP-mediated cytolysis (Mann & Whitney's *U*-test); NS: not significant.

ture. The product of the effective volume of the DPH probe by its shape factor, $V_e f$, has been taken as 1.7×10^{-22} cm^3 (Kawato, Kinosita & Ikegami, 1977). The wobbling diffusion constant D_w may be calculated by the relation given by Lipari and Szabo (1980)

$$D_w \cdot \phi \cdot (1 - r_\infty/r_0) = -X^2 \cdot (1 + X^2) \cdot \{\log[(1 + X)/2] + (1 - X)/2\}/ 2 \cdot (1 - X) + (1 - X) \cdot (6 + 8X - X^2 - 12X^3 - 7X^4)/24 \quad (9)$$

where $X = \cos \theta_c$.

The "viscosity in the cone," as noted by Kinosita et al. (1981), should be distinguished from the "microviscosity" (Shinitzky & Barenholz, 1978) which reflects largely the range of motion of the probe rather than the rate.

Temperature Dependence of the Fluorescence Polarization of DPH

Temperature scans were performed by cooling or heating at 1°C/min the cuve holder connected to a thermostat (Haake model FK). The temperature of the sample was monitored by a chromel-alumel thermocouple (Omega Eng., Stanford, Connecticut) placed into the sample, just above the excitation beam. Temperature data were obtained from a digital voltmeter (Systron multimeter 7344H) coupled to the 9835A Hewlett-Packard microcomputer. The temperature dependence of the anisotropy parameter, $(r_0/r - 1)^{-1}$ was determined over the range 10–60°C. The value of r_0 was taken as 0.39 (Kawato et al., 1977; Gallais et al., 1982). The neperian logarithm of the anisotropy parameter was plotted against the inverse of the absolute temperature, as previously described (Livingstone & Schachter, 1980).

Results and Discussion

The effects of DPPC SUV preincubation on the endotoxin-induced activation of macrophages for tumor cell lysis are reported in Table 1. When a mixed culture of macrophages and tumor cells was exposed to 1 mM DPPC SUV from 0 to 18 hr the cytolytic effect of macrophages induced by an incubation with endotoxins (10 μg/ml) from 18 to 30 hr was significantly enhanced. In the same way, exposure to DDPC SUV from 18 to 30 hr significantly increased the tumor cell lysis induced by macrophages incubated with endotoxins from 30 to 72 hr. On the contrary, DPPC SUV incubation from 0 to 18 hr had no effect on macrophage activation when endotoxins were added to the incubation medium after a lag period from 18 to 30 hr. DPPC SUV were without activating effect when added to the mixed culture of macrophages and tumor cells without subsequent addition of endotoxins. The reversibility of the DPPC SUV effect requires that DPPC SUV act just before the endotoxins which could imply a transient potentiation by DPPC SUV of the activating effect of endotoxins on macrophages.

This biological effect, which confirms previous results (Jeannin et al., 1985), is correlated here to a physical effect. This latter is evidenced by the modification of the fluorescence polarization of DPH embedded in the isolated fractions of membranes obtained either from the macrophages or from the tumor cells.

Upon observation by electron microscopy, the membranes isolated from macrophages appear to be rough endoplasmic reticulum (RER) without other subcellular organelles. The purity of the RER preparation was estimated better than 90% by morphological criteria (Fig. 1). The membrane isolation method used in our work was initially described for

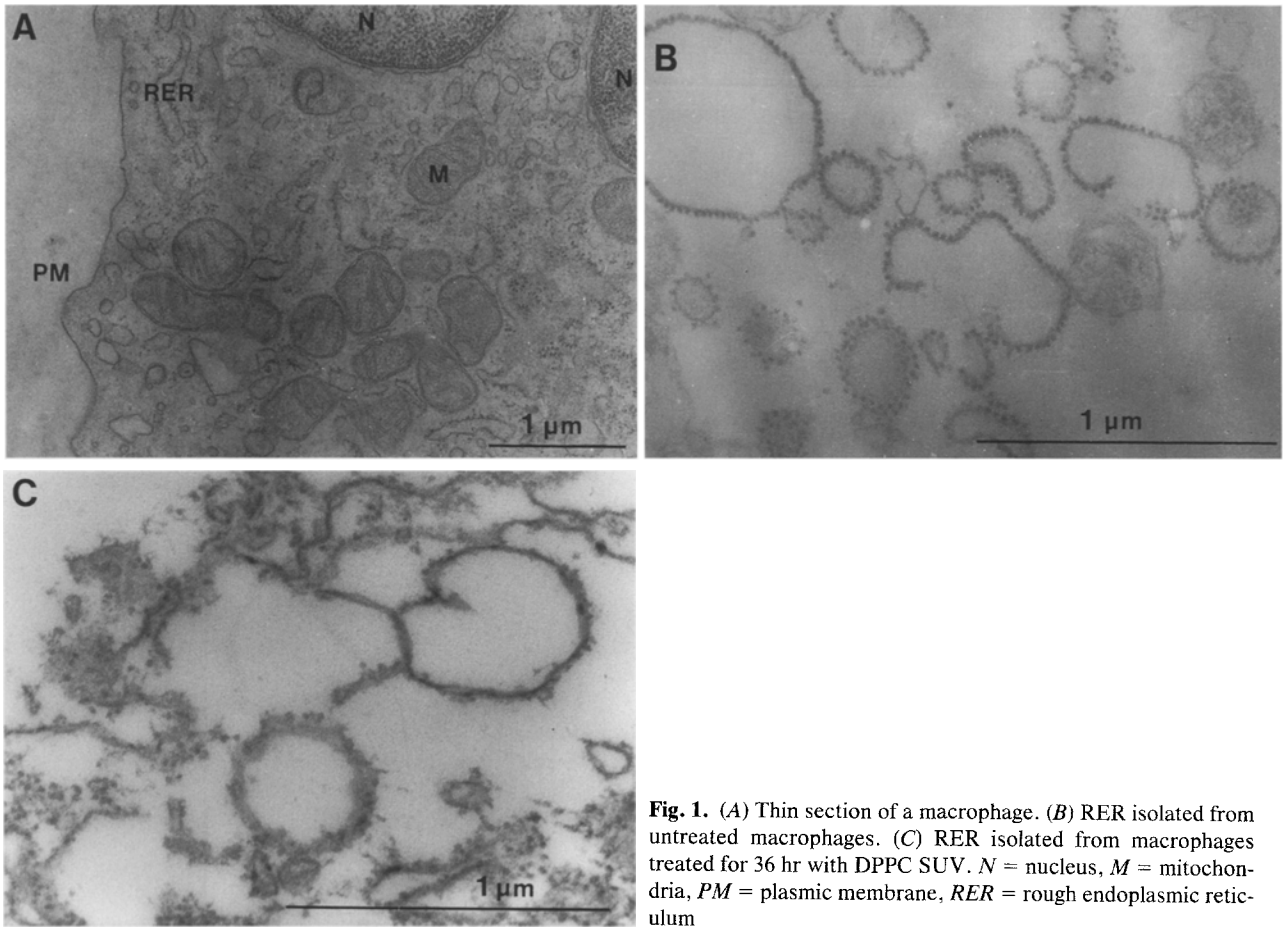

Fig. 1. (*A*) Thin section of a macrophage. (*B*) RER isolated from untreated macrophages. (*C*) RER isolated from macrophages treated for 36 hr with DPPC SUV. *N* = nucleus, *M* = mitochondria, *PM* = plasmic membrane, *RER* = rough endoplasmic reticulum

obtaining plasma cell membranes from mouse lymphocytes (Koizumi et al., 1981). The reason for which this method gives RER membranes when applied to rat macrophages has not been studied. When applied to rat colon cancer cells, this method gives membranes with the morphological appearance of both plasma and RER membranes when observed by electron microscope.

Figure 2 shows the fluorescence emission and excitation spectra of DPH embedded in the RER membranes isolated from macrophages and the background fluorescence of the membranes without DPH. After incorporation of DPH the excitation and emission maxima were at 360 and 430 nm, respectively. The spectrum excited at 280 nm before incorporation of DPH exhibited a maximum at 340 nm, i.e. the fluorescence emission of tryptophane in proteins.

The fluorescence intensity of the DPH probe measured at 37°C (excitation 360 nm, emission 430 nm) increased during the first 20 to 30 min after incorporation of the probe, then reached a plateau, whereas the fluorescence polarization value was constant during the total incorporation time (Fig. 3).

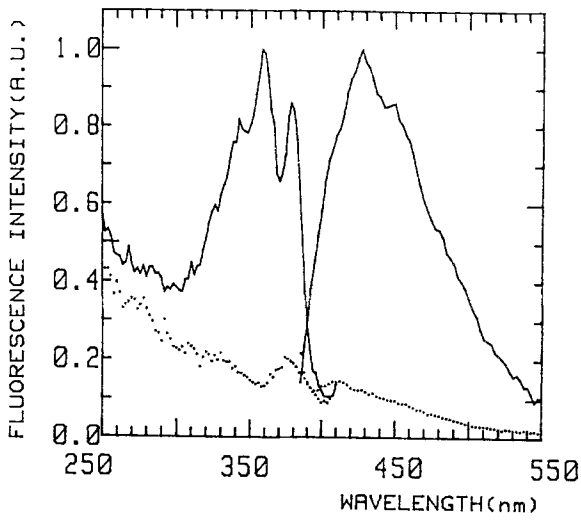

Fig. 2. Fluorescence excitation and emission spectra of RER membranes of macrophages with (—) or without (····) DPH. Excitation wavelength, 360 nm; emission wavelength, 430 nm. Bandwidths: excitation monochromator, 5.2 nm; emission monochromator, 12.8 nm

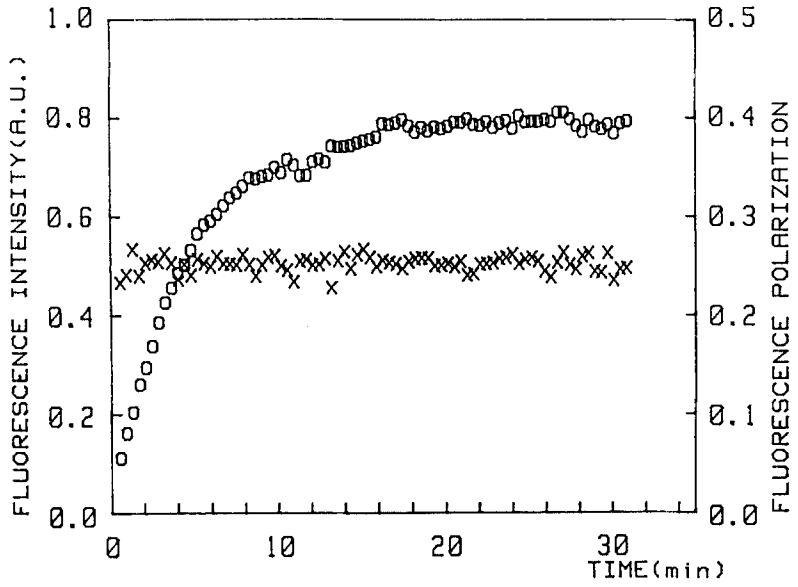

Fig. 3. Fluorescence intensity (×) and fluorescence polarization (○) of DPH as a function of the incorporation time of the probe. DPH (2×10^{-6} M) was incorporated in RER membranes of macrophages. Excitation wavelength, 360 nm; emission wavelength, 430 nm

Table 2. Fluorescence parameters of DPH in membranes isolated from cells treated or untreated with DPPC SUV[a]

Samples	r_s	r_∞	S	θ_c (deg)	θ (nsec)	D_w ($nsec^{-1}$)	η_c (P)	T_1 (nsec)	A_1	T_2 (nsec)	A_2	$\langle T \rangle$ (nsec)
RER	0.20	0.17	0.66	41	1.4	0.09	0.5	9.6	0.51	2.7	0.49	8.2
	(0.03)	(0.03)	(0.06)	(4)	(0.4)	(0.03)	(0.2)	(0.1)	(0.09)	(0.4)	(0.09)	(0.3)
RER-SUV	0.265	0.25	0.80	31	0.7	0.11	0.4	10.1	0.62	3.6	0.38	8.9
	(0.025)	(0.03)	(0.04)	(3)	(0.3)	(0.04)	(0.3)	(0.5)	(0.09)	(0.8)	(0.09)	(0.3)
TS	0.178	0.15	0.63	43	1.3	0.11	0.40	8.90	0.55	2.3	0.45	7.8
	(0.008)	(0.009)	(0.02)	(1)	(0.5)	(0.05)	(0.2)	(0.3)	(0.09)	(0.6)	(0.09)	(0.5)
TS-SUV	0.15	0.13	0.58	47	0.7	0.22	0.19	8.5	0.55	2.2	0.45	7.4
	(0.01)	(0.02)	(0.03)	(2)	(0.1)	(0.03)	(0.03)	(0.4)	(0.02)	(0.3)	(0.02)	(0.4)

[a] Fluorescence measurements at 37°C; r_s, steady-state anisotropy; r_∞, residual anisotropy; S, order parameter; θ_c, cone angle; ϕ, apparent relaxation time; D_w, wobbling diffusion constant; η_c, viscosity in the cone; T_1, A_1, T_2, A_2, fluorescence decay parameters for double-exponential decay; $\langle T \rangle$, average lifetime; RER, rough endoplasmic reticulum membranes isolated from untreated macrophages; RER-SUV, rough endoplasmic reticulum membranes isolated from macrophages treated with 2 mM DPPC SUV for 36 hr at 37°C; TS, membranes isolated from untreated tumor cells (K12/TS); TS-SUV, membranes isolated from tumor cells treated with 2 mM DPPC SUV for 36 hr at 37°C. Numbers in parentheses indicate the standard deviations for four independent determinations.

The steady-state anisotropy of DPH embedded in the RER membranes isolated from macrophages increased when the macrophages were treated with DPPC SUV (Table 2). This enhancement did not result from a decrease in fluorescence lifetime (Table 2). However, it was accompanied with an increase of the residual anisotropy, which reached 94% of the steady-state anisotropy (Table 2). This value clearly demonstrates that the "microviscosity" cannot be deduced from the steady-state anisotropy which reflects largely the static component rather than the dynamic one. The large increase in the order parameter leads to a wobbling cone angle $\theta_c = 31°$ (Table 2). The wobbling cone angle for DPH in other biological membranes ranges between 31 to 53° at 35°C (Kinosita et al., 1981). The values found here for the membranes of tumor cells and for the membranes of untreated macrophages are in the range of 41–47° (Table 2). The low value obtained for DPH in membranes isolated from macrophages treated with DPPC SUV could be the consequence of a large structural change of the membrane or, more exactly, of the microenvironment of the probe in the membrane.

This hypothesis was supported by the results of the measurement of the fluorescence anisotropy as a function of the temperature. Indeed, a phase transition appeared in the RER membranes of macrophages treated with DPPC SUV for 36 hr whereas in membranes isolated from untreated macrophages

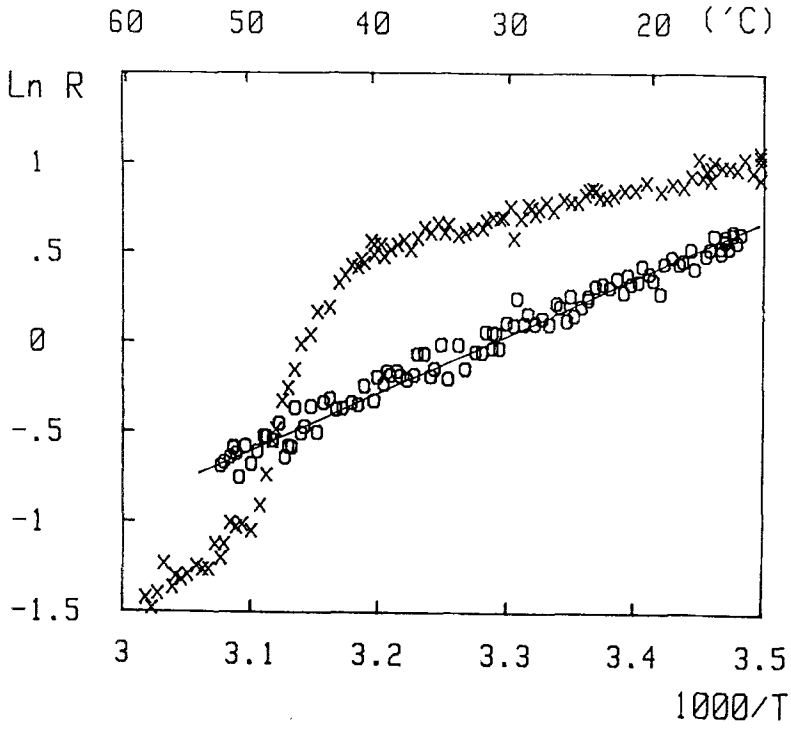

Fig. 4. Arrhenius plot of the anisotropy parameter of DPH embedded in RER membranes of macrophages. Ln R, neperian logarithm of the anisotropy parameter $R = 1/(r_0/r_s - 1)$ with $r_0 = 0.39$; T, absolute temperature. Macrophages were incubated with (×) or without (○) 2 mM DPPC SUV for 36 hr at 37°C

the neperian logarithm of the anisotropy parameter varied linearly with the inverse of the absolute temperature (Fig. 4).

An amplitude of the phase transition can be defined by the difference (Ln R_1 − Ln R_2), where Ln R_1 and Ln R_2 are the neperian logarithms of the anisotropy parameter at two temperatures, T_1 = 35°C and T_2 = 55°C, arbitrarily taken below and above the phase transition (Fig. 4). The amplitude of the phase transition increased with the time of the macrophage treatment with DPPC SUV. The phase transition occurred with a 12-hr treatment and was higher after a 24-hr or a 36-hr treatment (Table 2).

In the first heating scan the mid-point of the transition was 45 + 2°C (onset of the transition: 40°C, end: 50°C) which corresponded to a metastable state, as shown by the irreversibility of the temperature profile. However, from the second scan (cooling) a small reproducible hysteresis could be observed between further cooling and heating scans, leading to an average mid-point at 41.5 + 1°C (onset: 35°C; ed: 48°C, Fig. 5).

The microenvironment of the DPH probe appears to be in a gel phase at 37°C. A transition from a gel phase to a liquid crystalline phase is known for pure DPPC liposomes but is unusual in biological membranes. The average mid-point of the transition, T_m = 41.5°C, corresponds to that of pure DPPC in multilamellar vesicles (Lentz, Barenholz & Thompson, 1976) rather than to that of pure DPPC SUV for which T_m = 36 to 39°C (Jacobson & Papahadjopoulos, 1975, Lentz et al., 1976). The single unilamellar vesicles are unstable and are slowly transformed into a multilamellar structure (Suurkuusk et al., 1976). Liposomes which have contacted the cells may also be transformed into multilayered lipid aggregates (Margolis, 1984). Thus, the unusual phase transition observed could be due either to a contamination artefact or to a real structural modification of the membrane.

A contamination artefact could probably be ruled out from the following observations:

(1) No palmitic acid was detected by gas chromatography as methylester in the last washing of the RER membrane preparations submitted to a Folch extraction.

(2) The possibility that adsorbed DPPC SUV were transferred to RER preparation from plasma membranes during the cell homogenization was eliminated by the next experiment: macrophages were incubated with DPPC SUV for 36 hr, washed 3 times with complete medium, detached with a rubber policeman and incubated 15 min with trypsin 0.25% at 37°C. The macrophages were washed again then homogenized. Under these conditions the amplitude of the phase transition was not modified (Fig. 6).

(3) The phase transition was not obtained with the membranes isolated from tumor cells similarly treated with DPPC SUV (*see below*).

(4) An increase of palmitic acid was measured

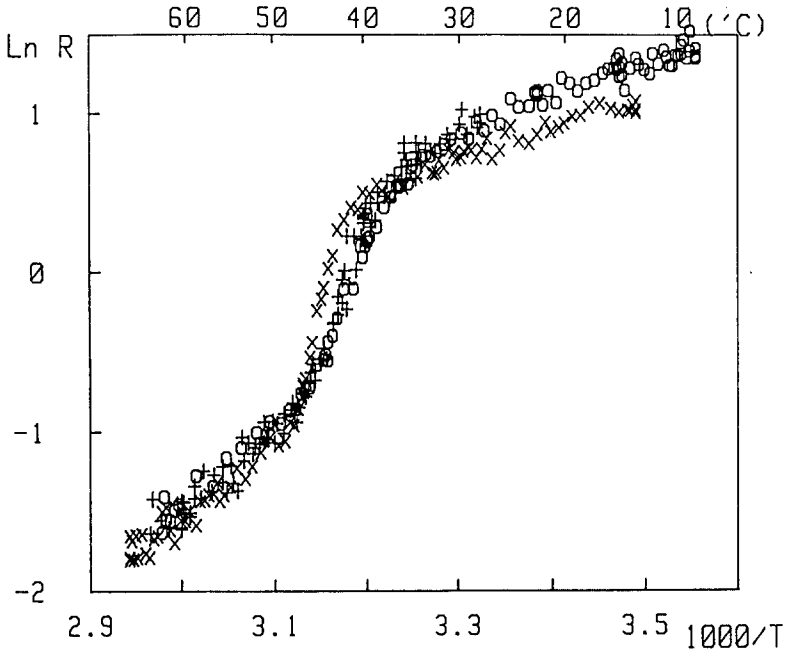

Fig. 5. Effect of successive temperature cycles upon the transition phase profile. DPH (2×10^{-6} M) was embedded in RER membranes of macrophages treated 36 hr at 37°C with 2 mM DPPC SUV. First scan, heating (×); second scan, cooling (○); third scan, heating (+). Coordinates as in Fig. 4

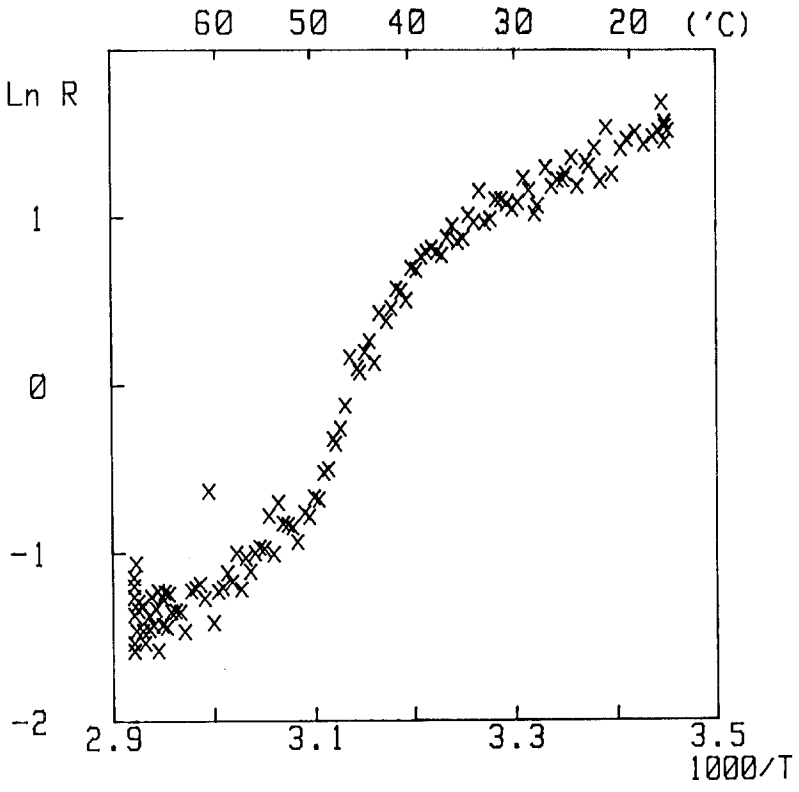

Fig. 6. Effect of trypsin on macrophages incubated with DPPC SUV. Macrophages were incubated with 2 mM DPPC SUV for 36 hr then washed, detached, treated 15 min with trypsin 0.25% at 37°C and washed again. DPH (2×10^{-6} M) was embedded in RER membranes, coordinates as in Fig. 4

by gas chromatography in the membranes isolated from macrophages but not in those isolated from tumor cells; both were treated with DPPC SUV (*results not shown*).

(5) The amplitude of the phase transition was incubation time-dependent (Table 3).

(6) The phase transition was reversibly suppressed in living macrophages (Table 3).

(7) No phase transition occurred when the macrophages were treated with DPPC SUV at 4°C instead of 37°C (Table 3).

Despite this latter observation, the endocytosis

Table 3. Appearance and disappearance of the phase transition induced by DPPC SUV treatment of macrophages[a]

Temperature, treatment and sequence duration	Phase transition	
0 12 15 24 36 hr	Presence	Amplitude
37°C————————————	No	
37°COOOOOOO	Yes	1.2
37°COOOOOOOOOOOOOOOOOOO	Yes	1.35
37°C————————!OOOOOOOOOOOOOO	Yes	1.7
37°COOOOOOOOOOOOOOOOOOOOOOOOOO	Yes	2.0
4°COOOOOOOOOOOOOOOOOOOOOOOOOO	No	
37°COOOOOOOOOOO! 37°C————————	No	
37°COOOOOOOOOOOOOOOOOO! 37°C————	No	
37°COOOOOOOOOOOOOOOOOO! 4°C————	Yes	2.2
4°COOOOOOOOOOOOOOOOOO! 37°COOOOOO	Yes	0.85
37°C×××××××××××! 37°C————————	No	
37°C×××××××××××! 37°COOOOOOOOOOOO	Yes	1.8
37°COOOOOOOOOOO! 37°C××××××××××××	Yes	1.3
37°C++++++++++++++++++! 37°C————	Yes	2.2
37°C++++++++++++++++++++++++++++	Yes	2.6
37°C●●●●●●●●●●●●●●●●●●●●●●●●●●	No	

[a] Membranes isolated from resident peritoneal macrophages incubated in complete medium (—) or with 2 mM DPPC SUV (OOO) or with 1 mM iodoacetic acid (×××) or with 2 mM DPPC SUV plus 1 mM iodoacetic acid (+++) for the given time at the indicated temperature, washed (!) then incubated in one of the above media, or treated with 10^{-5} M SUV of DPPC/cholesterol 1/1 mol/mol (●●●). Phase transitions are observed from the variation of the neperian logarithm of the anisotropy parameter of DPH in function of the inverse of the absolute temperature. Amplitude of the phase transition is defined as the difference ($LnR_1 - LnR_2$), where LnR_1 and LnR_2 are the neperian logarithms of the anisotropy parameter at two temperatures, $T_1 = 35$°C and $T_2 = 55$°C, arbitrarily taken below and above the phase transition.

of DPPC SUV cannot be at the origin of the phase transition since DPPC SUV also induced a phase transition in the RER membranes isolated from either dead macrophages or macrophages treated in the presence of iodoacetic acid (Table 3). This shows that the induction of the phase transition was a passive phenomenon. Moreover, endocytosis is a fast phenomenon (Petty & McConnell, 1983) whereas the phase transition appeared in the RER membranes only after a 12-hr treatment of macrophages with DPPC SUV and was at a maximum after 36 hr (Table 3).

The appearance of the phase transition in the RER membranes isolated from macrophages treated with DPPC SUV is a slow and passive phenomenon, which suggests a slow translocation of DPPC through the plasma membrane of the cell to the RER membranes. This hypothesis is supported by the lack of phase transition when the macrophages were treated by DPPC SUV at 4°C (Table 3), probably because plasma membranes are then too rigid to allow the translocation.

The phase transition observed involves the presence of DPPC domains in the RER membranes. Such domains, if not due to endocytosis or contaminant DPPC SUV, could be created by fusion, adsorption or phase separation. Fusion or adsorption of liposomes are passive phenomena (Huang, Ozato & Pagano, 1978; Poste & Papahadjopoulos, 1978). Fusion of pure diacetyl phospholipid SUV with membranes occurs with liposomes which are both fluid and negatively charged (Poste & Papahadjopoulos, 1978); DPPC SUV are not fluid but rather metastable at 37°C (Papahadjopoulos et al., 1973; Poste, 1980); furthermore, they adsorb to membranes at this temperature (Sandra, Paltzer & Thomas, 1981). Therefore, steady adsorption of DPPC to RER membranes, resistant to washing, could be at the origin of the phase transition. However, a modification of the composition of the membrane leading to a phase separation cannot be ruled out. It could be a consequence of the high concentration of DPPC used in the experiments; when the concentration of DPPC was 0.5 mM or smaller no phase transition was found. The phase separation results in the coexistence of clusters of gel and liquid-crystalline lipids in a membrane (Chapman, Urbina & Keough, 1974), with a phase transition at a

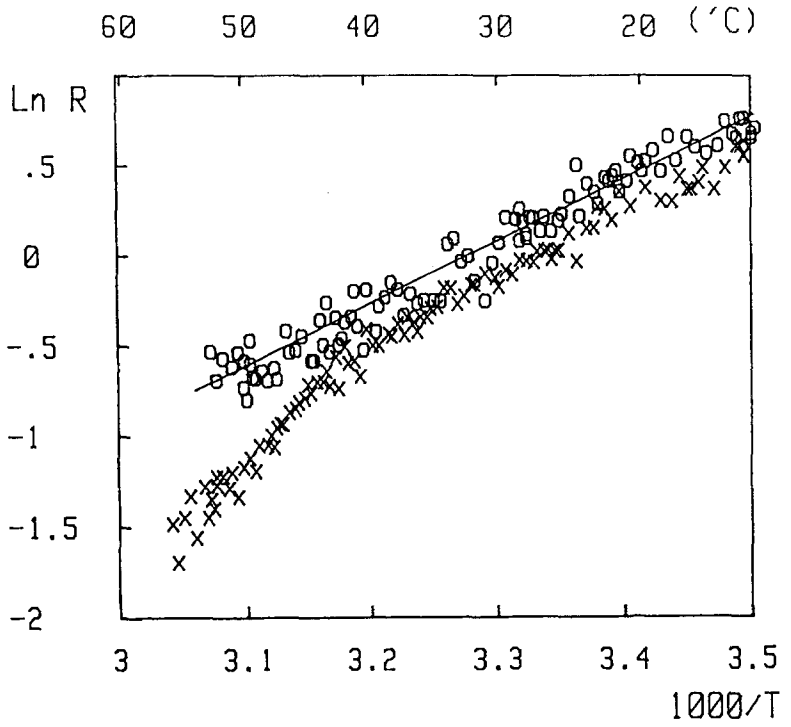

Fig. 7. Arrhenius plot of the anisotropy parameter of DPH embedded in membranes of tumor cells. Tumor cells were incubated with (×) or without (O) 2 mM DPPC SUV for 36 hr at 37°C. Coordinates as in Fig. 4

temperature equal to the transition temperature of the pure gel lipid (Jacobsen & Papahadjopoulos, 1975).

The phase transition disappeared when DPPC SUV was ulteriorly removed from the culture medium (Table 3). This reversion was an active phenomenon observed with living macrophages, only in the absence of iodoacetic acid and not with already dead macrophages (Table 3). This active cellular reversion, which occurred within 12 hr at 37°C, but did not take place at 4°C (Table 3), could be due either to the lysis of the adsorbed liposomes or to the degradation of the DPPC microdomains and to the turnover of the RER membranes.

The treatment of tumor cells by DPPC SUV led to effects quite different from those observed with macrophages. Steady-state and residual anisotropies of DPH were not enhanced but slightly lowered (Table 2). No phase transition could be detected; only a break point in the slope of the Arrhenius plot of the anisotropy parameter was found. This thermotropic transition was not present in the membranes of untreated tumor cells (Fig. 7). The slope for untreated tumor cells was similar to the one obtained with membranes of untreated macrophages; an apparent activation energy of 6.5 + 0.5 kcal/mol was estimated. Values for apparent activation energy of DPH in biological membranes from 4.8 to 6.5 kcal/mol have been measured (Livingstone & Schachter, 1980).

The rotational correlation time of DPH and the "viscosity in the cone" were lowered in membranes isolated from DPPC-treated tumor cells, whereas the order parameter was only slightly modified (Table 2). The membranes isolated from tumor cells treated with DPPC SUV seem affected rather in their fluidity than in their structure. They became more fluid at 37°C, which could be a consequence of cholesterol depletion. DPPC liposomes are known to remove cholesterol from cells (Kramers et al., 1980). The removal of cholesterol could lead to the coexistence of fluid and rigid microdomains in membranes (Brasitus, Tall & Schachter, 1980; Livingstone & Schachter, 1980). This could account for the break point appearing in the Arrhenius plot of the anisotropy parameter of DPH in membranes isolated from tumor cells treated by DPPC SUV (Fig. 7).

Addition of cholesterol (C) to DPPC inhibited the SUV effect on the macrophage-mediated cytolysis induced by endotoxins. Treating macrophages and tumor cells with 1 mM SUV of C/DPPC (1/1; mol/mol) from 0 to 18 hr then washing them prior to addition of 10 μg endotoxins/ml from 18 to 30 hr failed to modify the macrophage-mediated cytolysis. No phase transition could be observed in the RER membranes isolated from macrophages treated with 1 mM SUV of C/DPPC (1/1; mol/mol) from 0 to 36 hr (Table 3).

There seems to be a correlation between the synergistic effect of DPPC SUV on the activation of macrophages by endotoxins (biological effect) and

the appearance of a phase transition in the RER membranes isolated from macrophages treated by DPPC SUV (physical effect). These two effects were transient and they had similar kinetics of induction and reversion. The addition of cholesterol to DPPC inhibited both the biological and the physical effects. Neither effect was apparently induced by endocytosis. The membrane preparations isolated from macrophages were identified by electron microscopy as rough endoplasmic reticulum without other subcellular organelles. However, the change observed in the structure of the RER membranes of the treated macrophages has not been proved to be the cause of the synergy.

Up to now, very little is known about the subcellular events that underlay macrophage activation. Endotoxins encapsulated into liposomes (phosphatidylcholine/phosphatidylserine, 7/3, MLV) activate macrophages to a tumoricidal state (Dijkstra et al., 1987). An equal subliminal amount of free endotoxins in the presence of control liposomes is without effect. This indicates that the endotoxins act at an intracellular level. It could be supposed that DPPC SUV adsorbed to the macrophage cell surface enhance the binding of endotoxins and their internalization and hence stimulate the activation. This mechanism was proposed for the synergistic effect of liposomes on the B lymphocyte proliferation induced by endotoxins (Ozato, Huang & Pagano, 1978). However, such a mechanism is not specific. This synergistic effect is obtained with SUV composed of dipalmitoyl lecithin, of dimyristoyl lecithin or of egg yolk lecithin/cholesterol, 1/1. Furthermore it is obtained when liposomes are incubated with lymphocytes either at 22 or 2°C. The synergistic effect of liposomes and endotoxins on the activation of macrophage tumoricidal activity is specific of DPPC SUV and obtained only when they are incubated with macrophages at 37°C (Jeannin et al., 1985). Therefore a nonspecific effect of liposomes on the binding and the internalization of endotoxins cannot be the only cause of their synergistic effect on the macrophage activation. Macrophages are able to produce a cytolytic peptide, called tumor necrosis factor (TNF). While TNF mRNA synthesis occurs constitutively, the peptide is not translated under normal circumstances. Endotoxins have been shown to greatly increase the rate of TNF gene transcription and to allow the post-transcriptional phase of biosynthesis to proceed (Beutler et al., 1986). Cytolytic macrophages show a decrease rate of rRNA synthesis (Varesio, 1985). The acquisition of cytolytic activity induced by endotoxins and lymphokins (Meltzer, 1981) is associated with a decreased accumulation of mature 28 S rRNA compared to 18 S rRNA (MacKay & Russel, 1986). This decrease is reversible since activated macrophages that lose their cytolytic activity again accumulate 28 S rRNA (MacKay & Russel, 1986). These observations involve a functional modification at the rRNA level; it is tempting to link this to the observed structural modification of the RER. The above-mentioned studies and our present results suggest that the amplification by DPPC SUV of the tumoricidal activity of macrophages triggered by endotoxins could be related to modifications of both the macrophages, but more data is needed to elucidate the mechanism(s) involved.

The authors thank Prof. F. Martin and Prof. M. Duquesne for their interest, Dr. A. Bernard and A. Hammann for their help in the characterization of membranes by electron microscopy, and L. Ramirez who revised this manuscript.

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Received 25 August 1987; revised 28 April 1988