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The plasma-membrane internalization and recycling is enhanced in macrophages upon activation with gamma-interferon and lipopolysaccharide; a study using the fluorescent probe trimethylaminodiphenylhexatriene

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The lipophilic fluorescent probe trimethylaminodiphenylhexatriene (TMA-DPH), previously used as a plasma membrane marker in membrane fluidity and exocytosis studies, was shown, to monitor the plasma-membrane internalization-recycling shuttle movement in cells. Using this approach we present here kinetic and dose-response data, which give evidence that the plasma membrane flow is enhanced in bone marrow macrophages from various mouse strains, upon in vitro activation with gamma interferon (IFN-γ) or bacterial lipopolysaccharide (LPS), within physiological dose ranges. The effect studied evolved in line with the usual development kinetics of macrophage activation. Complementary assays on membrane fluidity, surface charge density and membrane surface indicated no related changes. From these experiments it is concluded that the observed enhancement of the plasma membrane traffic does not originate from specific limited membrane modifications, but is merely a particular feature of the overall macrophage activation.

Introduction

In the preceding paper in this issue it was shown that the lipophilic fluorescent probe trimethylamino-diphenylhexatriene (TMA-DPH) [1], used in membrane fluidity (see for instance, Refs. 2–4) and exocytosis [5,6] studies could also monitor the typical plasma-membrane internalization-recycling flow in L929 cultured mouse fibroblasts and in mouse bone-marrow macrophages. After rapid incorporation into the plasma membrane, the probe remains associated with this membrane, throughout its two-way intracellular flow, and is thus conveyed towards lysosomes. The TMA-DPH fluorescence intensity kinetic data were consistent with the features of this process, previously described by other groups [7–10] using horseradish-peroxidase as fluid-phase marker and [^{3}H]galactose as covalent membrane label. The plasma-membrane flow rate was found to be 3–4-times higher in macrophages than in L929 cells, matching earlier results [11]. This obviously suggested a correlation between the membrane flow intensity, and the level of metabolic activity, and raised the question of whether cell stimulation with appropriate effectors would result in a parallel increase in the membrane flow. This question seemed particularly interesting in macrophages, in view of the considerable attention paid to their activation mechanisms and, above all, to their tumoricidal capacities (for reviews, see Refs. 12–15). However, to our knowledge, little information was so far available on that subject [16,17]. We present here a first series of results showing that in bone-marrow macrophages from several mouse strains the TMA-DPH internalization or exocytosis rate is enhanced after in

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DPH, diphenylhexatriene; IFN-γ, gamma interferon; LPS, lipopolysaccharide; PBS, phosphate-buffered saline (without Ca^{2+}, Mg^{2+}); TMA-DPH, trimethylamino-diphenylhexatriene.

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vitro activation by gamma interferon (IFN-γ) or bacterial lipopolysaccharide (LPS). The effect was found to be dose-dependent, and it suggested a novel approach of macrophage activation.

Materials and Methods

Cell cultures

Bone-marrow-derived macrophages were obtained by culturing femora stem cells from 6–8-week-old Balb/c or C57BL/6 or CBF1 male mice. CBF1 is a cross of the first two. The conditions were described in the preceding paper. The cells were seeded in a glucose-enriched DMEM medium with 10% foetal calf serum, 5% horse serum and 25% L929-conditioned supernatant. They were kept in a 8% CO_2 atmosphere, at 37 °C, in teflon bags, and harvested between day 8 and 11 at a yield of $(1.0–1.2) \cdot 10^6$ cells/ml.

Macrophage activation treatments

Macrophages were activated in vitro with IFN-γ (Holland Biotechnology, The Netherlands) and/or LPS (from *Salmonella abortus equi,* prepared [18] and generously provided by Dr. C. Galanos, Max-Planck Institut für Immunbiologie, Freiburg, F.R.G.). In kinetic studies, these substances were introduced directly into the teflon culture bags, respectively, 16 and 8–10 h before the experiments, at routine doses of 50 or 100 U/ml and 1 μg/ml. In dose-responses studies, it was more conveneint to add them to adherent macrophages layers conditioned in 6-well dishes, as described below (kinetic internalization assays).

Macrophage activation control assay

The level of macrophage activation was routinely controlled by the production of O_2^- superoxide anions, which was determined by a lucigenin-dependent chemiluminescence assay [19] with a Biolumat 9505 chemiluminometer (Berthold, Wildbad, F.R.G.). $2 \cdot 10^5$ cells were allowed to adhere for 2 h at 37 °C in chemiluminescence vials (Berthold) in serum-free medium. After removal of the medium, chemiluminescence was measured in 0.5 ml of Hepes-buffered Eagle's medium containing 250 μM lucigenin (Boehringer, Mannheim). After 5 min pre-incubation, the process was triggered by the addition of zymosan (type A, Sigma) at a final concentration of 5 mg/ml. The count rate per min was recorded at 1 min intervals.

Kinetic TMA-DPH internalization and exocytosis assays

The experiments were performed as described in the preceding paper. $5 \cdot 10^5$ cells were allowed to adhere for 2 h at 37 °C in 6 wells, 35 mm in diameter culture clusters (Costar) in 2 ml DMEM/10% foetal calf serum. The layers were washed and re-incubated, at 37 °C, with the same medium containing $2 \cdot 10^{-6}$ M TMA-DPH (molecular Probes, Oregon, U.S.A.) for various times. The layers were then washed five times for about 3 s with 2 ml Ca^{2+}-Mg^{2+}-free PBS (PBS) at room temperature, which ensured extraction of the probe from the plasma membrane without changing the internalized fraction. This was a consequence of the partition equilibrium of TMA-DPH [2,5] due to its amphiphilic character. The fluorescence intensity was measured with a MPF 66 Perkin-Elmer Spectrofluorimeter after recovering the cells by gentle scraping with a 'cell lifter' (Costar) (excitation 360 nm; emission 435 nm). The results were calibrated with reference to the fluorescence intensity of TMA-DPH incorporated in the surface membrane, assessed by measurements without cell washing, after a short incubation with PBS containing TMA-DPH at the same concentration.

In exocytosis kinetic studies, the cells were pre-incubated with TMA-DPH ($2 \cdot 10^{-6}$ M) at 37 °C, as described above, for selected times, and then washed and re-incubated, at 37 °C, in TMA-DPH-free medium with 10% foetal calf serum, for various times. The fluorescence intensity was measured after washing once with PBS.

Fluorescence anisotropy measurements

Membrane fluidity was studied by fluorescence anisotropy measurement, to determine whether or not macrophage activation elicited important changes in the plasma membrane composition or structure. To that end, macrophage suspensions were centrifuged at low speed and the pellet resuspended in PBS, at a density of $2 \cdot 10^5$ cells/ml. 1 ml aliquots from this cell suspension were rapidly mixed with 1 ml of a TMA-DPH ($2 \cdot 10^{-6}$ M) solution in PBS, in quartz cuvettes. The fluorescence anisotropy,

$$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$$

which conceptually describes membrane fluidity in terms of decreasing hindrance of rotational motion [20], was measured every 5 s with an SLM 8000 spectropolarofluorimeter, as described previously [2]. $I_{\parallel}$ and $I_{\perp}$ represent the components of the fluorescence intensity, respectively, parallel and perpendicular to the vertically polarized excitation light.

Results

Macrophage activation enhances the internalization and exocytosis of TMA-DPH

The TMA-DPH internalization kinetics (Fig. 1) display a biphasic aspect, as reported in the preceding paper. The rapid increase in fluorescence intensity in the first phase was attributed to pinocytosis of external TMA-DPH solution and the further gradual increase to pinosome-lysosome fusion, according to earlier studies

with horseradish-peroxidase as a marker [7,8,10]. Treating the macrophages with 50 U/ml IFN-γ, 16 h before the assay, or with 1 μg/ml LPS, 10 h before, increased both parts of the curves. The effect was more pronounced for Balb/c than for C57BL/6 macrophages, whereas for macrophages from CBF1, i.e., a strain derived from the previous two, the effect was intermediate.

Exocytosis kinetics were enhanced too (Fig. 2a). The initial rapid decrease in fluorescence intensity was longer and more rapid for treated cells than for controls. The second part of the curve, which accounts for the lysosomal staining, also reflected the effect of the activation, indicating that both IFN-γ, and LPS had enhanced TMA-DPH incorporation in lysosomes. The influence of LPS was relatively low in this particular experiment. All the results correlated well with those of the lucigenin-dependent chemiluminescence assay (Fig. 2b) performed in parallel. Moreover, when L929 mouse fibroblasts were used as negative control, neither IFN-γ nor LPS induced any increase in TMA-DPH internalization or exocytosis.

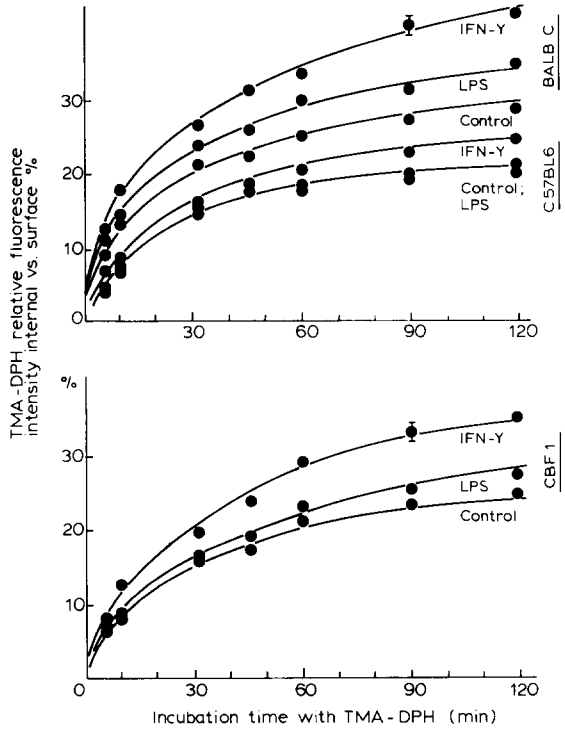

Fig. 1. The effect of macrophage activation with IFN-γ or LPS on TMA-DPH internalization in bone-marrow macrophages from various mouse strains. The cells were treated with 50 U/ml IFN-γ or 1 μg/ml LPS, respectively, 16 and 10 h before the assay. After cell adherence in 6-well culture clusters ($2.5 \cdot 10^5$ cells/ml) the macrophages were incubated at 37°C with TMA-DPH $2 \cdot 10^{-6}$ M in DMEM/10% foetal calf serum. The fluorescence intensity was measured after thorough cell washing and gentle scraping in 2 ml PBS (excitation 360 nm; emission maximum 435 nm). The results were expressed as % of TMA-DPH fluorescence intensity in the plasma membrane, measured in unwashed cells after 1 min incubation with TMA-DPH at the same concentration in PBS. The points represent the mean from six determinations and the error bars, typical S.D.

TABLE I

The dose response for TMA-DPH internalization in Balb/c bone marrow macrophages after treatment with IFN-γ and LPS

$2.5 \cdot 10^5$ cells/ml were incubated, after adherence, in 6-well culture clusters, in 2 ml DMEM/10% foetal calf serum with $2 \cdot 10^{-6}$ M TMA-DPH, for 45 min at 37°C. After thorough cell washing and gentle cell scraping, the fluorescence intensity (F.I.) at 435 nm (excitation 360 nm) was measured with a MPF 66 Perkin-Elmer spectrofluorimeter. IFN-γ and LPS were added, respectively, 16 h and 10 h before the assay. The results were expressed as % of TMA-DPH fluorescence intensity in the plasma membrane, measured in unwashed cells after 1 min incubation with TMA-DPH at the same concentration in PBS. The reported values denote mean and standard derivation from six determinations.

Relative fluorescence intensity at 435 nm internal vs. surface (%)					
IFN-γ		LPS		IFN-γ + LPS	
U/ml	F.I.	μg/ml	F.I.	U/ml + μg/ml	F.I.
0	16±1	0	16±1	0	16±2
20	21±1	0.25	17±2	20; 0.25	24±3
50	23±1	1.0	20±1	20; 1.0	30±3
100	26±1	10.0	29±2	50; 1.0	26±3
500	19±2				

The effects on the TMA-DPH internalization in Balb/c bone-marrow macrophages of varying the IFN-γ and the LPS doses are summarized in Table I. The internalized fluorescence intensity, measured after 45 min of incubation, increased with that of the stimulation, except for very high doses (e.g., 500 U/ml IFN-γ). Moreover, IFN-γ and LPS exhibited synergistic effects, although each of them alone could elicit the same maximum effect. This maximum, corresponding to an almost 100% increase in fluorescence intensity, could be achieved, for example, with 100 U/ml IFN-γ or 10 μg/ml LPS, or 20 U/ml IFN-γ + 1 μg/ml LPS.

The delays in the effect of macrophage activation on TMA-DPH internalization following the treatment were characteristic of the activation process [12,13]. The effect of IFN-γ only became appreciable after 12 h and had leveled off after 18 h, indicating that protein synthesis was needed. For LPS, the effect began after 3 h, being maximum after 10 h.

Complementary data

Fluorescence anisotropy data, for TMA-DPH incorporated into the plasma membrane of Balb/c bone-marrow macrophages, at various doses of IFN-γ and LPS and treatment times are shown in Table II. The results denote the absence of any membrane fluidity effect resulting from the treatment. This conclusion differs from earlier studies [21] on inflammatory peritoneal macrophages, but they were performed with diphenylhexatriene (DPH), a fluorescent label, without any specificity for the plasma membrane.

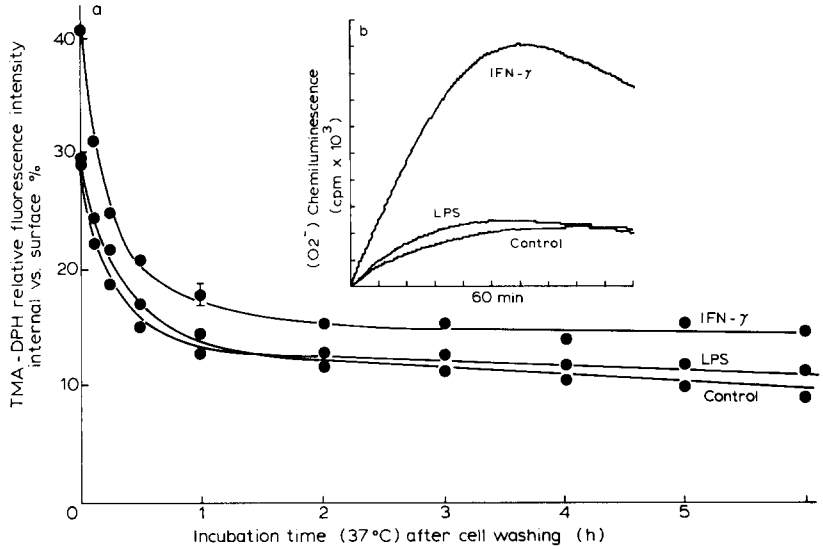

Fig. 2. (a) TMA-DPH exocytosis kinetics in activated and control Balb/c macrophages. Cells ($2.5 \cdot 10^5$/ml) were first incubated at 37°C for 60 min with TMA-DPH ($2 \cdot 10^{-6}$ M) in DMEM/10% foetal calf serum and then washed and re-incubated with TMA-DPH free medium. The fluorescence intensity at 435 nm (excitation 360 nm) was measured after washing and recovery in 2 ml PBS, at various time intervals. The macrophages were treated with 50 U/ml IFN-γ and 1 μg/ml LPS, respectively, 16 h and 10 h before assay. The points represent the mean from six determinations and the error bar, typical S.D. (b) Correlative lucigenin-dependent chemiluminescence assay for O_2 production. $2 \cdot 10^5$ macrophages were adhered in serum-free medium in vials designed for chemiluminescence. The chemiluminescence signal was then followed in 0.5 ml Hepes-buffered Eagle's medium containing 250 μM lucigenin, after the addition of 5 mg/ml zymosan to trigger the process.

Nor was any difference detected after activation, when membrane surface potential was investigated with the potential-sensitive fluorescent dye bis-oxonol [22], used at a final concentration of 100 nM, as described in [23].

No change in the surfaces of the macrophage plasma membrane was induced either by the activation. The surfaces were assessed from the TMA-DPH fluorescence intensity after short incubation (< 1 min) without cell washing. Under these conditions, the fluorescence intensity reflects only the plasma-membrane labelling and is proportional to the plasma membrane surface [2,5,24]. The validity of this approach depends on no drastic change in membrane composition or charge density resulting from the activation treatment, leading to changes in TMA-DPH partition equilibrium. This could probably be assumed, in view of the above results on membrane fluidity and membrane potential. Typical fluorescence intensity values obtained in this way were 195 ± 10 (S.D.), 168 ± 9, 176 ± 8, 165 ± 9 for, respectively, Balb/c, C57BL/6, CBF1 macrophages and L929 cells. The measurements were performed at 435 nm

TABLE II

TMA-DPH fluorescence anisotropy values in the plasma membrane of Balb/c bone marrow macrophages before and after treatment with IFN-γ and LPS

1 ml cell suspension in PBS ($2.5 \cdot 10^5$ cells/ml) was added to 1 ml TMA-DPH $2 \cdot 10^{-6}$ M in a quartz cuvette and the fluorescence anisotropy was measured after approx. 1 min incubation with a SLM 8000 spectropolarofluorimeter. The values denote mean ± S.D. from six separate determinations.

	Fluorescence anisotropy value (×1000)							
	Control		IFN-γ 100 U/ml		LPS 1 μg/ml		IFN-γ 50 U/ml + LPS 0.25 μg/ml	
Time after treatment	1 h	16 h	1 h	16 h	1 h	8 h	1 h	16 h
	282 ± 5	283 ± 5	285 ± 4	281 ± 5	286 ± 4	282 ± 4	285 ± 5	280–5

emission maximum, on suspensions of $2.5 \cdot 10^5$ cells/ml with TMA-DPH $2 \cdot 10^{-6}$ M. Pretreatment with 100 U/ml IFN-γ or 1 μg/ml LPS did not modify these values, within the limits of experimental error.

Discussion

The biphasic trend of TMA-DPH internalization kinetics in cells was discussed in the preceding paper of this issue. The probe is taken up from the external solution by the formation of plasma membrane pinocytic vesicles. For homeostatic reasons, vesicle influx has to be balanced by returning vesicles, but, at the beginning of the incubation with TMA-DPH, very few of the latter are labelled, and the resulting fluorescence intensity increases rapidly. When more returning vesicles are labelled, the effect slows down (the fluorescence is lost in the aqueous external medium) and finally levels off when the flow of labelled vesicles is equal in both directions. The further residual increase (second part of kinetic curves) is interpreted by the fusion of TMA-DPH labelled vesicles with lysosomes. The fluorescence intensity increases continuously because the fluorescence intensity of the vesicles leaving lysosomes is negligible compared to that of those entering into fusion with them, as a consequence of TMA-DPH partition equilibrium. Similar comments could be made as to the interpretation of TMA-DPH exocytosis kinetics.

Fig. 1 shows that when macrophages were activated with IFN-γ or LPS, the TMA-DPH internalization kinetics were enhanced in both part of the curve. From the relative increase in the slopes it could be inferred that both fluid-phase pinocytosis and fusion with lysosomes were enhanced. TMA-DPH exocytosis curves (Fig. 2) led to the same conclusions.

The effect observed is specifically correlated with macrophage activation:

(i) control experiments with non-activable L929 cells displayed no change in the TMA-DPH internalization features after IFN-γ treatment;

(ii) the effect studied appeared within delays matching the kinetics of macrophage activation by IFN-γ and LPS in vitro and for similar dose ranges of these agents [13];

(iii) TMA-DPH internalization and exocytosis features were parallel to the production of O_2^- superoxide anions, assayed by lucigenin-dependent chemiluminescence. This was even true for the 'inhibitory' effect observed for very high doses of IFN-γ (500 U/ml).

The effect did not result from any immediate change in the plasma membrane structure, induced by IFN-γ or LPS: neither the membrane fluidity, nor the surface charge, nor the surface were changed.

As regards the plasma membrane surface, the result might be surprising since it has been reported [17] and it is frequently observed that adherent macrophages become bigger after activation with IFN-γ; however, this corresponds rather to the enhancement of adherence properties.

Finally the enhancement of the plasma membrane flow in activated macrophages, which was easily monitored with the fluorescent probe TMA-DPH, emerges as merely another aspect of the overall 'basal stage' of macrophage activation. This is borne out by the responsiveness of the mouse strains studied, which indicates that genetic factors are involved and suggests that the mechanisms concern the cell machinery as a whole. Possible correlations with the development of tumoricidal properties have not yet been investigated, but are scheduled, on the basis of thorough dose-response studies.

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