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# Membrane changes in lipopolysaccharide-stimulated murine B lymphocytes associated with cell activation

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The lateral diffusion of the fluorescent lipid analog 3,3′-dioctadecylindocarbocyanine iodide (DiI) was measured in the membranes of murine B lymphocytes treated with the B cell mitogen lipopolysaccharide (LPS). The mobility of DiI, as measured by fluorescence photobleaching recovery (FPR) techniques, was temperature-dependent with a value of $6.1 \cdot 10^{-9}$ $cm^2 s^{-1}$ at 37°C. Untreated cells exhibited this diffusion coefficient over 72 h in culture. In contrast, DiI mobility decreased to $2.0 \cdot 10^{-9}$ $cm^2 s^{-1}$ at 37°C in membranes of LPS-stimulated lymphocytes 24 h following LPS exposure. Interestingly, this decreased lipid lateral diffusion was not accompanied by any change in surface immunoglobulin lateral diffusion which remained essentially unchanged at $3.6–4.3 \cdot 10^{-11}$ $cm^2 s^{-1}$ over 72 h. To determine whether LPS effects on lipid lateral diffusion were due to insertion of LPS into the cell plasma membrane, we examined TRITC-LPS diffusion in B lymphocytes from LPS-responsive Balb/c and C3Heb/FeJ mice and from hypo-responsive C3H/HeJ mice. DiI and TRITC-LPS mobility decreased more than 50% in LPS-stimulated Balb/c and C3Heb/FeJ cells by 72 h. On C3H/HeJ lymphocytes, there was no change in DiI or TRITC-LPS lateral diffusion throughout the incubation period. These data indicate that B lymphocyte membrane composition is altered in LPS-activated lymphoblasts and that the decreased lateral diffusion of lipid probes does not result from membrane perturbation by LPS insertion into the lipid bilayer. Further, similarities between TRITC-LPS and DiI lateral diffusion suggest that most LPS molecules interact non-specifically with B cell membranes, presumably by acyl chain insertion of the lipid A moiety.

## Introduction

Lipopolysaccharide (LPS) from Gram-negative bacteria causes mitogenic stimulation of a variety of murine cell types including B lymphocytes [1]. The lipid A backbone of LPS has been shown to be responsible for the mitogenic activity of LPS [2] which is mediated through plama-membrane components. Jakobovits et al. [3] have demonstrated that LPS response occurs in normally unresponsive B cells following fusion of membrane vesicles from responder mouse B cells to LPS-nonresponsive cells. Glycoprotein and glycolipid receptors [4,5] as well as surface immunoglobulin [6] have been implicated as LPS binding membrane constituents. More recently, LPS-binding proteins have been identified on murine macrophage-like cells [7]. However, the well documented affinity of LPS for biological and artificial membranes [8], as well as studies showing non-specific interactions of LPS with hepatocyte membranes [9], point to an initial interaction between LPS and the lipid bilayer which may be distinct from a receptor-mediated signalling event.

LPS-induced B-lymphocyte maturation offers an opportunity to examine interactions of LPS with plasma membranes and the changes in membranes associated with blastogenesis. That membrane organization changes following antigenic or mitogenic stimulation is indicated by a number of membrane associated changes including plama-membrane depolarization [10], enhanced I-A expression [11], redistribution of surface proteins [12] and reduced membrane fluidity [13]. Changes in B cell membrane composition during differentiation into mature plasma cells may be important in the efficient response of B lymphocytes to immunoregulators from other cells of the immune system [14].

Membrane fluidity, as measured by the lateral mobility of membrane constituents, provides a useful quantitative indicator of changes in membrane compo-

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Abbreviations: DiI, 3,3′-dioctadecylindocarbocyanine iodide; LPS, lipopolysaccharide; FPR, fluorescence photobleaching recovery; TRITC, tetramethylrhodamine isothiocyanate; BSS, balanced salt solution; FCS, fetal calf serum; PBS, phosphate-buffered saline.

sition. The laser optical technique of fluorescence photobleaching recovery permits measurement of lipid or protein lateral diffusion in the membranes of single cells under physiological conditions. We have used this technique to examine lipid lateral mobility on LPS responsive (C3Heb/FeJ) and non-responsive (C3H/HeJ) murine B lymphocytes and LPS interactions with the plasma membrane in order to understand better the plasma-membrane events associated with LPS-mediated mitogenesis.

## Materials and Methods

*Chemicals.* Roswell Park Memorial Institute (RPMI) 1640 tissue culture medium supplemented with 200 mM glutamine, penicillin, and streptomycin were obtained from KC Biological (Lenexa, KS). Fetal bovine serum (FCS; Lot 1111550) was purchased from Hyclone (Logan, UT). *E. coli* lipopolysaccharide 0128 : B12 (LPS), tetramethylrhodamine isothiocyanate-labeled 0128 : B12 LPS, tetramethylrhodamine isothiocyanate (TRITC), goat anti-mouse IgM, Ficoll, and gentamicin were obtained from Sigma (St. Louis, MO). Rabbit anti-mouse T cell antiserum was purchased from Accurate Chemical and Scientific (Westbury, NY). Guinea pig serum was purchased from Cedarlane Labs (Hornby, Ontario). The lipid analog 3,3'-dioctadecylindocarbocyanine iodide (DiI) was synthesized by the method of Sims et al. [15].

*Preparation of TRITC-Fab fragments of mouse anti-IgM antibodies.* To prepare TRITC-Fab fragments of anti-mouse IgM antibodies, goat anti-mouse IgM (2 mg) was bound to 100 mg protein A Sepharose by incubation in phosphate buffer (pH 7.5) for 30 min at room temperature. The Sepharose beads were washed extensively by centrifugation. TRITC (10 mg) was dissolved in 5 ml of dimethylsulfoxide and diluted to 20 ml with bicarbonate buffer (pH 9.0), and 2.4 ml of this was added to the Sepharose. The solution was stirred overnight at 4°C or for 3 h at room temperature. The beads were washed and to them was added 200 $\mu$l papain (0.1 mg/ml in 0.01 M phosphate (pH 7.4) containing 0.15 M NaCl, PBS), 50 $\mu$l cysteine HCl (0.2 M in PBS), and 400 $\mu$l PBS. This solution was incubated for 2 h at 37°C with occasional mixing. Iodoacetamide (100 $\mu$l of 0.4 M) and 50 ml of 0.1 M $MgCl_2$ were added to quench the reaction. The Sepharose beads were pelleted and the supernatant was dialyzed against Tris-buffered saline (pH 7.4) overnight at 4°C. The solution was extracted with 5 ml of butanol to remove excess rhodamine. The aqueous solution contained approx. 0.1 mg/ml protein, with a TRITC-to-protein molar ratio of 0.23.

*Thymidine incorporation.* Murine lymphocytes were diluted to a concentration of $2.5 \cdot 10^6$ cells/ml in RPMI 1640 supplemented with 5% fetal bovine serum, $2 \cdot 10^{-3}$ M glutamine, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and 2-mercaptoethanol at a final concentration of $5 \cdot 10^{-5}$ M. LPS, Con A and TRITC-LPS were used at a concentration of 10 $\mu$g/ml. Cells were dispensed in Costar 96 well microtiter dishes at $4.5 \cdot 10^5$ cells/well using sterile techniques. The total incubation volume was 200 $\mu$l. Incubations were performed for 48 h in 5% $CO_2$ in air at 37°C. At 18 and 42 h, 1 $\mu$Ci [$^3$H]thymidine was added to each well. After 6 h cells were collected and washed on glass fiber filters using a MASH II harvester (Microbiological Association, Bethesda, MD). The filters were dried at room temperature and placed in 4 ml scintillation fluid (0.4% Omnifluor-25% Triton X-114-xylene). Samples were counted using a Beckman LS 7500 scintillation counter with a 41% counting efficiency for $^3$H (Beckman Instruments, Palo Alto, CA).

*Lymphocyte preparation.* The mice used in these experiments were 6–8 week old female Balb/c from Charles River Laboratories, Wilmington, MA, and C3Heb/FeJ and C3H/HeJ from Jackson Laboratories, Bar Harbor, MA. The mice were killed by cervical dislocation. The spleens were removed and placed in cold balanced salt solution (BSS). Cells were removed by gentle teasing of the spleen and the debris allowed to settle. The cells were transferred to a sterile tube and washed twice with BSS by centrifugation at $500 \times g$ for 10 min. The splenocytes were cultured in RPMI 1640 supplemented with 5% FCS, 2 mM L-glutamine, penicillin (100 IU/ml), streptomycin (100 $\mu$g/ml) and gentamicin (100 $\mu$g/ml) at a concentration of $5 \cdot 10^6$ cells/ml in 25-cm$^2$ flasks (Corning No. 25100) at 37°C in a 5% $CO_2$ atmosphere. Cell cultures were supplemented daily with 0.1 ml of RPMI 1640 containing 33% FCS.

The experiments performed on unstimulated or small B-cells required the removal of T-cells since B- and T-cells cannot be distinguished visually. The cells from a single spleen were incubated in 15 ml BSS containing 0.75 ml rabbit anti-mouse T-cell antiserum for 60 min at 4°C. Cells were centrifuged at $500 \times g$ for 10 min and resuspended in 13 ml BSS with 2 ml guinea pig complement. The viable cell number following this treatment was reduced by approx. 50%, as determined by trypan blue exclusion. Diffusion experiments on differentiating B-cells were performed following stimulation by 10 $\mu$g/ml LPS in the presence of $5 \cdot 10^{-5}$ M 2-mercaptoethanol.

To prepare cells for fluorescence photobleaching recovery measurements, the cells were pelleted by centrifugation at $500 \times g$ for 10 min and then suspended in BSS containing 0.1% sodium azide for 30 min at 37°C. The cells were washed once with BSS and resuspended in 1 ml of BSS. DiI (10 $\mu$l of 0.1 mg/ml DiI in 95% ethanol) was added while gently vortexing. Cells were incubated for 10 min at 4°C, washed twice with

BSS, and resuspended in 0.5 ml of BSS. To fluorescently label sIg, $10^6$ cells in 1 ml BSS were incubated with 50 $\mu$l TRITC-Fab solution (0.5 mg/ml) on ice for 1 h. The procedure for labeling with TRITC-LPS was similar to labeling with DiI except that cells were incubated for 45 min at 37°C following the addition of 50 $\mu$l of 1 mg/ml TRITC-LPS in BSS. Prior to use in cell labeling, the TRITC-LPS solution was sonicated for 5 min and then centrifuged in a Beckman Airfuge (40 000 $\times g$ for 5 min).

*Fluorescence photobleaching recovery measurements.* The FPR system used in these experiments has been described in detail elsewhere [16]. FPR measurements were performed at 37°C under coverslips on well slides using a Zeiss Universal microscope equipped with a thermally-controlled stage, III/RS fluorescence vertical illuminator, and a 50 × water immersion fluorescence objective. An attenuated beam of a Coherent Radiation 90–3 argon ion laser operating at 514.5 nm was focused on the upper plasma membrane of the cell. The beam $1/e^2$ radius was $4.8 \cdot 10^{-5}$ cm. Emitted fluorescence was isolated by a standard filter set used for tetramethylrhodamine. Molecules within a 1 $\mu m^2$ beam region were bleached by a 5-ms pulse of 2 mW 514.5 nm light. The laser was then attenuated 20 000-fold and fluorescence from the bleached area was measured as unbleached, fluorescent DiI or TRITC-LPS molecules diffused into the bleached region. Fluorescence was measured by a thermionically cooled C31034A photomultiplier tube operated in a single photon counting mode. A photobleaching run on a single cell consisted of 128 fluorescent measurements to establish the prebleach fluorescence, a bleaching pulse, and additional measurements, up to 32 768 total, to delineate fluorescence recovery kinetics. Measurements of DiI and LPS diffusion were taken at 20 ms per point. Bleaching pulses were 5 ms and 8 ms for DiI and TRITC-LPS, respectively. Unless otherwise indicated, all measurements were made with the thermally controlled stage maintained at 37°C. Data acquisition and system functions were controlled by an IBM PC/AT computer. Data were analyzed on-line using optimized non-linear curve fitting procedures to obtain the diffusion coefficient of the mobile fluorophore and the percent of the fluorescence recovery.

## Results

### *Temperature dependence of DiI mobility in LPS-stimulated cells*

The diffusion of DiI on LPS-stimulated and unstimulated Balb/c lymphocytes was measured over the range of 10–37°C at 0, 24, 48, and 72 h. DiI mobility on unstimulated cells increased monotonically with increasing temperature, increasing 6-fold from $1.0 \cdot 10^{-9}$ $cm^2 s^{-1}$ at 10°C to $6.1 \cdot 10^{-9}$ $cm^2 s^{-1}$ at 37°C (Fig. 1, upper panel). At 24 h (middle panel) and 72 h (lower panel) following LPS exposure the mobility of DiI at 37°C was dramatically different in differentiating, relative to unstimulated, lymphocytes. The diffusion coefficient of DiI on LPS-treated cells was greatly decreased at 37°C, but less so at the intermediate and low temperatures examined. These results indicate that the changes in membrane composition which alter membrane fluidity occur during LPS-induced blastogenesis. Despite decreases in lipid diffusion, there was no change in the lateral diffusion of TRITC-labeled goat anti-mouse IgM Fab fragments bound to membrane IgM. The membrane IgM diffusion coefficients remained relatively constant at $4 \cdot 10^{-11}$ $cm^2 s^{-1}$ over the known timecourse of lymphocyte differentiation (Table I). Fluorescence recovery after photobleaching was approx. 50% at all time points, which corresponds well to values previously reported [17].

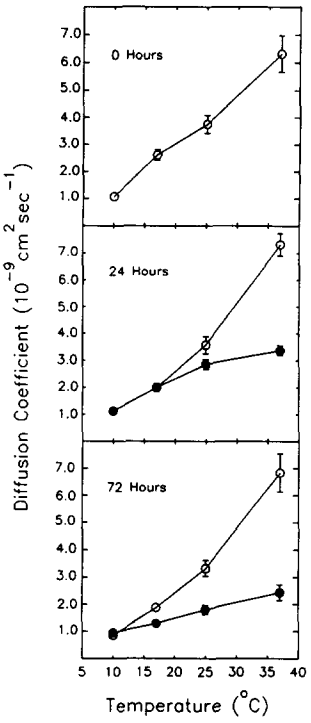


Fig. 1. DiI mobility as a function of temperature on LPS-stimulated (●) and resting (○) Balb/c B cells cultured for various times. Cells were incubated with 10 $\mu$g/ml LPS for the indicated periods. Fluorescence photobleaching recovery measurements were made at the indicated temperatures by the use of a thermionically controlled microscope stage. For measurements of DiI-labeled B cells at 0 h (upper panel), T cells were removed using anti-mouse T-cell serum and guinea-pig complement. At 24 h (middle panel) and 72 h (lower panel), lymphoblasts were identified visually. Results are the mean ± S.E. of at least 10 measurements on individual cells.

### *TRITC-LPS diffusion on Balb/c lymphocytes*

The lateral mobility of TRITC-LPS and DiI on Balb/c B cells was measured at various times after stimulation by LPS (Fig. 2). Measurements at 0 h were made on spleen cell suspensions in which T cells had

TABLE I

*Diffusion of TRITC anti-μ Fab fragments on B cells cultured with LPS*

TRITC goat anti-μ-chain Fab fragments were prepared as described in Materials and Methods. Murine spleen cells were stimulated with 10 μm/ml LPS for the indicated times. Cells were suspended in 1 ml BSS with 50 μl TRITC-Fab for 1 h on ice. Values given are the means of 20–30 measurements on different cells. Fluorescence recovery after photobleaching was approx. 50% in all cases.

| Hours in culture | Cell type | Diffusion coefficient ($10^{-11}$ cm$^2$ s$^{-1}$) |
|---|---|---|
| 0 | lymphocyte | 3.6±0.1 |
| 24 | lymphoblast | 4.3±0.2 |
| 48 | lymphoblast | 4.1±0.3 |
| 73 | lymphoblast | 4.1±0.1 |

been removed by complement-mediated cytolysis. Direct microscopic examination was used to distinguish lymphoblasts from resting B cells in spleen cell cultures. At 24, 48 and 72 h the basis of this distinction was increased cell size and decreased nuclear-to-cytoplasmic ratio [1]. Cells ($5 \cdot 10^6$ per ml) were incubated with 50 μg/ml TRITC-LPS for 45 min at which time all cells were labeled. The lateral diffusion coefficient of DiI decreased progressively from a 0 h value of $3.8 \cdot 10^{-9}$ cm$^2$ s$^{-1}$ to $1.7 \cdot 10^{-9}$ cm$^2$ s$^{-1}$ at 72 h. TRITC-LPS displayed a similar decrease in mobility with time with a 0 h diffusion coefficient of $2.6 \cdot 10^{-9}$ cm$^2$ s$^{-1}$ and $1.6 \cdot 10^{-9}$ cm$^2$ s$^{-1}$ by 72 h. Recovery of fluorescence after photobleaching in these, and all subsequent, experiments was > 70% for TRITC-LPS and > 85% for DiI. That the TRITC-LPS preparation retained full biological activity can be seen from the [$^3$H]Thy uptake experiments measuring the activation and proliferation of C3Heb/FeJ B cells following TRITC-LPS exposure (Fig. 3, lower panel). As expected, C3H/HeJ B cells did not respond to TRITC-

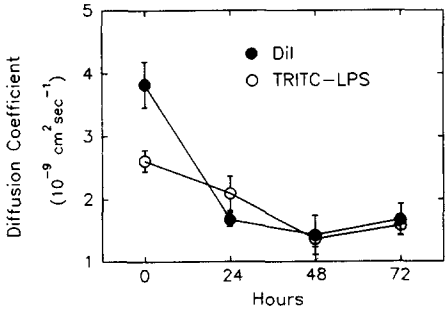


Fig. 2. DiI and TRITC-LPS mobility on LPS-stimulated Balb/c B cells cultured for various times. Cells were incubated with 10 μg/ml LPS for the indicated times. For FPR measurements at 0 h, T cells were removed using anti-mouse T cell serum and guinea pig complement. At 24, 48 and 72 h, FPR measurements were made on B lymphoblasts identified visually. Measurements were made using a temperature-regulated microscope stage maintained at 37°C. Results are the mean ± S.E. of at least 15 measurements on individual cells.

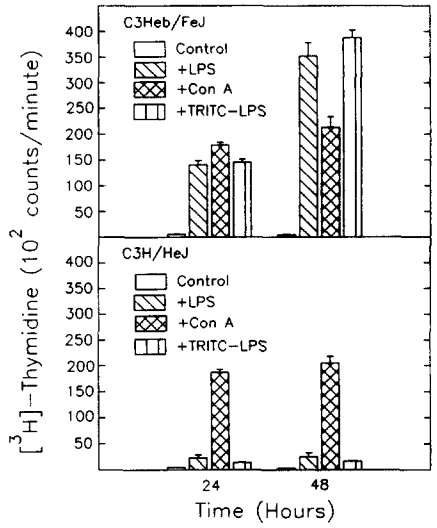


Fig. 3. Mitogenic activity of TRITC-LPS with non-responsive C3H/HeJ and responsive C3Heb/FeJ lymphocytes. C3Heb/FeJ (upper panel) and C3H/HeJ (lower panel) spleen cell cultures were dispensed into 96 well microtiter dishes at $4.5 \cdot 10^5$ cells/well. LPS, TRITC-LPS, and Con A were added to a final concentration of 10 μg/ml. 1 μCi [$^3$H]thymidine was added to each well 6 h before harvesting the cells. Proliferation was determined by [$^3$H]thymidine incorporation, as described in Materials and Methods. Results are the mean ± S.E. of six measurements for each treatment.

LPS (Fig. 3, lower panel). T cells from both mouse strains responded to Con A.

*TRITC-LPS diffusion on C3H/HeJ and C3Heb/FeJ lymphocytes*

The decrease in lipid lateral diffusion in LPS-stimulated B lymphocytes might arise from membrane perturbation following insertion of LPS into the bilayer. To determine whether the decrease in lipid lateral diffusion was caused by such LPS insertion or arose during blast transformation, the lateral mobility of DiI and TRITC-LPS was examined on B cells from LPS-responsive (C3Heb/FeJ) and non-responsive (C3H/HeJ) mouse strains (Fig. 4, upper and lower panels, respectively). The mobility of both DiI and TRITC-LPS on C3Heb/FeJ lymphocytes decreased progressively over the 72 h incubation period. On C3H/HeJ cells, the lateral mobility of DiI and TRITC-LPS was not affected by LPS exposure. DiI diffusion coefficients on cells which had not been exposed to LPS maintained 0 h values throughout the 72 h incubation period, indicating that the changes in lipid mobility did not arise from culture conditions (data not shown).

## Discussion

Our results suggest that extensive changes in membrane composition may occur following LPS activation of B cells, as indicated by the temperature-dependent decreases in the diffusion coefficients for DiI in LPS-

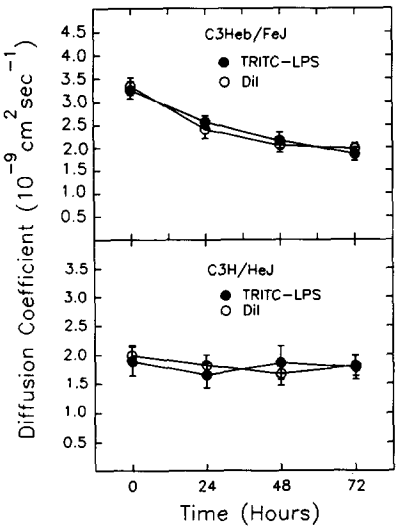


Fig. 4. DiI and TRITC-LPS mobility on LPS-treated non-responsive C3H/HeJ and responsive C3Heb/FeJ B cells cultured for various times. Cells were incubated with 10 $\mu$g/ml unlabeled LPS for the indicated times. For measurements on C3Heb/FeJ (upper panel) cells at 0 h and C3H/HeJ (lower panel) cells at all time points, T cells were removed with anti-mouse T cell serum and guinea pig complement as described in Materials and Methods. Cells were labeled for 45 min at 37°C with 50 $\mu$g/ml TRITC-LPS. All measurements were made using a temperature-regulated microscope stage maintained at 37°C. The mean ± S.E. of at least 15 measurements are reported.

treated lymphoblasts (Fig. 1). Given the highly lipophilic nature of the lipid A moiety of LPS and the affinity of lipid A for artificial and biological membranes, it was conceivable that the decrease in lipid mobility in LPS-stimulated B cells might result from perturbation of the lipid bilayer by lipid A insertion, as suggested by the fact that TRITC-LPS has lateral diffusion characteristics similar to the phospholipid analog DiI in Balb/c B cells (Fig. 2). By examining the diffusion characteristics of TRITC-LPS and DiI on LPS responsive (C3Heb/FeJ) and hypo-responsive (C3H/HeJ) murine B cells, membrane changes associated with cell proliferation or LPS binding alone could be observed. These results clearly show that the decrease in lipid lateral diffusion occurs on LPS-treated lymphoblasts (Fig. 4). TRITC-LPS bound to C3H/HeJ cells and had a diffusion coefficient identical to that of TRITC-LPS bound to LPS-responsive C3Heb/FeJ cells at time zero. However, these non-responsive cells did not display the decreased lipid lateral diffusion following LPS treatment exhibited by the C3Heb/FeJ cells.

The lateral mobility of TRITC-LPS on the LPS responsive and hypo-responsive murine B cells has also provided additional insight into LPS interactions with B cell membranes. In all of the mouse strains tested, including both LPS-responsive and non-responsive mice, TRITC-LPS diffused at rates reported for membrane lipids, $4\text{–}15 \cdot 10^{-9}$cm$^2$ s$^{-1}$. This, and the fact that its rate of diffusion is slower on blast cells which have more viscous membranes [13], indicates that most, if not all, TRITC-LPS molecules interact non-specifically with B cells. Values for TRITC-LPS lateral diffusion are in good agreement with those reported for fluorescein isothiocyanate-LPS on rat hepatocytes and mouse neuroblastoma cells using FPR [18], $4.0 \cdot 10^{-9}$ cm$^2$ s$^{-1}$ and $1.0 \cdot 10^{-8}$ cm$^2$ s$^{-1}$, respectively.

The post-bleach fluorescence recovery of TRITC-LPS was typically 70–85%, while DiI fluorescence recovery, on the other hand, was 90–95%. These differences in fluorescence recovery suggest that a small fraction of TRITC-LPS are immobile over the time scale of the measurements. TRITC-LPS may form aggregates on the surface of lymphocytes, which, if sufficiently large, would appear immobile and indeed LPS has been shown to self-associate in phospholipid bilayers [8]. Alternatively, a fraction of TRITC-LPS molecules may be bound to membrane proteins either specifically or non-specifically. Differences in glycolipid composition between C3Heb/FeJ and C3H/HeJ mice suggest the involvement of glycolipids in LPS binding and transmembrane signalling [5]. LPS has been reported to bind to a number of cell surface proteins including I-A/I-E histocompatibility antigens [19] and surface immunoglobulin [12] although in our hands membrane IgM lateral diffusion is both an order of magnitude slower than TRITC-LPS and not affected by significant changes in the plasma membrane (Table I). Alternatively, the differences in DiI and TRITC-LPS mobility in Balb/c lymphocytes at 0 h might reflect TRITC-LPS partitioning into membrane domains quite different from those preferred by DiI [8].

LPS binding via intercalation of the acyl chains of lipid A into the plasma membrane is consistent with the non-specific and non-saturable binding kinetics observed by other investigators [20]. Using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene and fluorescent polarization techniques, Portolés et al. [21] have shown that the addition of LPS to rat hepatocyte suspensions induces an increase in membrane microviscosity and a delay in the fall of microviscosity induced upon heating indicative of non-specific LPS binding to hepatocyte membrane. Knowing the number of TRITC-LPS molecules bound to a cell under optimally mitogenic LPS concentrations allows speculation about the TRITC-LPS population being measured. By comparing the photon emission from a TRITC-LPS labeled Balb/c B cell with that of a cell-sized latex bead containing a known number of TRITC molecules, we calculate that approx. $2\text{–}4 \cdot 10^8$ TRITC-LPS molecules are bound to each B cell under optimally mitogenic conditions, a number considerably greater that the typical number of protein receptors expressed on a cell surface. It can be inferred from the amount of TRITC-LPS bound, and from the rapid rate of diffusion of TRITC-LPS on both LPS-responsive and non-responsive B cells, that the

majority of bound TRITC-LPS is *not* associated with membrane proteins. However we cannot rule out the possibility that short-lived interactions with slowly diffusing proteins results in a slower apparent diffusion coefficient for the lipid probe.

However, it is quite possible that a receptor-mediated signalling event is being masked by the non-specific binding of TRITC-LPS to the cell membrane. A two-step mechanism for LPS binding and subsequent transmembrane signal transduction has been previously proposed by Morrison [22], in which a non-specific binding via lipid A intercalation into the lipid bilayer is followed by a signalling event through a membrane-associated protein. Alternatively, as suggested by Wright et al. [23], physiological effects of LPS may depend on interaction with a circulating LPS-binding protein followed by receptor-medicated response. Neither of these mechanisms precludes additional non-specific LPS interactions with the plasma membrane.

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