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MEMBRANE PERTURBATION OF MACROPHAGES STIMULATED BY BACTERIAL LIPOPOLYSACCHARIDE

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SUMMARY: Macrophages recovered from regressing Moloney sarcomas lose their capacity to kill tumor cells when held 12-24 h in culture. Exposure of this population to low concentrations (ng/ml range) of bacterial lipopolysaccharide will cause a rapid resurgence of cytolytic activity, however. By using electron spin resonance techniques and either 5- or 12-doxyl stearate spin labels, we have shown that exposure of cultured tumor macrophages to lipopolysaccharide (100 ng/ml) is followed by plasma membrane perturbation. The change was most pronounced 3-4 h after stimulation, and had subsided within 5-6 h. The relationship of the described perturbation to the induction of killing cannot be determined from these studies; however, neither cytolytic activity nor the membrane change developed in other kinds of macrophages exposed to similar concentrations of lipopolysaccharide.

Among the protean effects of bacterial lipopolysaccharide is the capacity to "activate" macrophages (1,2). The activated state is operationally defined here as that in which macrophages acquire the capacity to kill tumor cells. Little is known about the sequence of events that leads to activation. It is likely, however, that the process begins after a stimulus is applied to the cell surface. We decided, therefore, to search for early changes in the plasma membrane of macrophages exposed to lipopolysaccharide. The system in which we chose to work was one in which macrophage activation (i.e., expression of cytolytic activity) can be modulated predictably and consistently (3).

Macrophages isolated from spontaneously regressing Moloney sarcomas are able to kill neoplastic cells when freshly explanted but, after 24 h in vitro, lose their cytolytic

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activity entirely (4). It is the regressor macrophage held 24 h in culture that is the essential element in the studies to be described here. This cell, which we have referred to as "primed", is functionally unique because minute amounts (pg-to-ng/ml) of bacterial lipopolysaccharide (endotoxin) will trigger in it the sudden resurgence of high levels of killing activity (3). In contrast to primed macrophages, thioglycollate-elicited peritoneal macrophages cannot be stimulated to kill by similar concentrations of lipopolysaccharide (3). Thus, by choosing the source of macrophages and the time and conditions of their culture, we were able to analyze functionally homogeneous macrophage populations that differ with regard to their capacity to kill neoplastic cells.

Because of its well documented usefulness for studying the structure and function of biological membranes (for current review, see ref.5), we employed the electron spin resonance technique. The results we report here show: (i) that there is a transient perturbation of the plasma membranes of primed macrophages exposed to lipopolysaccharide, (ii) that no similar change occurs in the plasma membranes of thioglycollate-elicited peritoneal macrophages or of various other cell types exposed to similar concentrations of lipopolysaccharide, and (iii) that this change precedes the onset of detectable tumor cell killing. The observed membrane perturbation may be part of the mechanism through which lipopolysaccharide mediates at least one of its effects.

METHODS AND MATERIALS

Regressing Moloney sarcomas were induced in mice, harvested aseptically 11 days later, and disaggregated enzymatically (6). As described before (3,4) macrophages were isolated by repeated (3-to-4), short term (10 min each) exposures of cells in the crude suspensions to the bottoms of wells in 24-place plastic plates (Costar, Cambridge, Mass.) This plating procedure yielded an adherent population of cells that numbered 10^6/well, as estimated by direct microscopic counting (3). Of these, 85-90% were macrophages, judging from their morphologic appearance and their ability to phagocytose zymosan particles. The other cell type consistently found in monolayers -- usually 5% of the total -- was the MSC cell (7) used to initiate the sarcomas. Monolayers held in culture for 24 h were incubated in HEPES-buffered (15 mM) Eagle's minimum essential tissue culture medium containing 30% fetal bovine serum. In some experiments macrophages were fixed (30 min) in glutaraldehyde (0.025%) and washed several times with phosphate buffered saline before exposing them to lipopolysaccharide.

Peritoneal macrophages were harvested by peritoneal lavage 4-5 days after 3 ml Brewer's thioglycollate broth had been injected intraperitoneally. Monolayers were established by allowing peritoneal exudate cells to settle either on the bottoms of plastic wells or on the inner surfaces (front and back) of glass microslides (Vitro Dynamics, Rockaway, N.J.). Non-adherent cells were removed from monolayers by repeated, vigorous washings, leaving a population comprised of 95% macrophages.

Cell lines were passaged routinely in medium containing 10% serum. The continuously-cultured macrophage cell line, P388D1, was a gift from Dr. Hillel S. Koren (Department of Immunology, Duke University, Durham, N.C.). All other lines were on hand in our laboratory. Purified lipopolysaccharide isolated from phenol-extracted Escherichia coli (0111:B4) was obtained from Dr. David C. Morrison of the Research Institute of Scripps Clinic. The preparation and quantification of this reagent has been described (3).

As before (3,4), the amount of chromium-51 (^{51}Cr) released from prelabeled P815 mastocytoma cells in 16 h was used to indicate macrophage-mediated cytolysis. This assay measures killing, as has been previously determined by correlation with light and scanning electron microscopic examination of target cells, and by cell counting in long term (48 h) cultures. Plasma membranes were spin labeled and electron spin resonance spectra were recorded as described in the legend for Fig. 1. Order parameters were calculated as described by Gaffney (8).

RESULTS

It first was imperative to establish that spin labels were incorporated into the lipid bilayer of plasma membranes, rather than merely adsorbed onto cell surfaces. Fig. 1 shows that the spectra for 5-doxyl and 12-doxyl stearate were different. Such a difference is consistent with the labels being located within a flexibility gradient in a membrane. Adsorption of the two spin labels to the cell surface would have produced identical spectra for the two labels. As was evidenced by the absence of spin-spin interaction, the labels seemed to be dispersed randomly and not clustered. The spectra are characteristic for anisotropicly immobilized fatty acid spin labels in a viscous environment (10). We believe that the labels were located in the plasma membranes of macrophages. This statement is based on the following observations: (a) Kaplan et al. (11) reported that fatty acid spin labels are reduced readily in membranes of nucleated cells but that the labels can be reoxidized by treating cells with membrane impermeant ferricyanide ions. These results indicate that the labels were located predominantly in the plasma membrane. We have found similar properties of labels introduced into

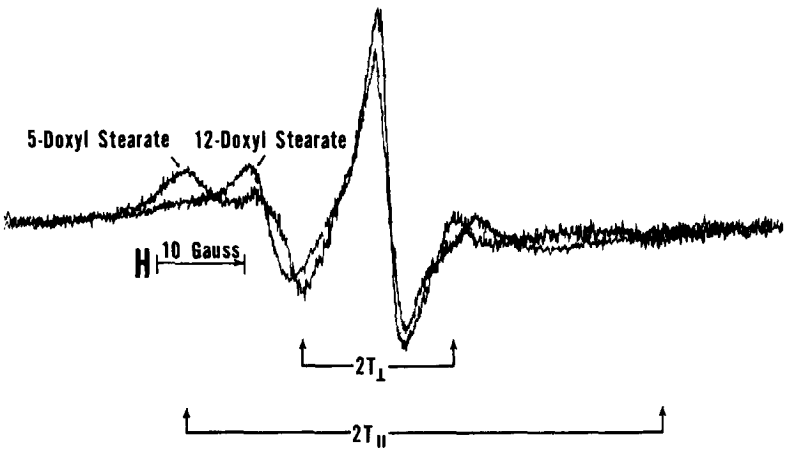

Figure 1. Electron spin resonance spectra of two different spin labels incorporated into membranes of primed macrophages. For labeling, adherent cell types were scraped (rubber policeman) or jetted (Pasteur pipette) free from their substratum, washed by centrifugation (5 min, 4°, 600 g_{max}) and resuspended in 100 µl of ice cold phosphate buffered saline. The final cell concentration was usually 2×10^7/ml. Spin label (either 5- or 12-doxyl stearate from SYVA, Palo Alto, Calif.) was dissolved in ethanol (125 µg/ml). Seven µl of this solution were added to the chilled cell suspension. After 10 min the cells were diluted to 2 ml with ice cold saline and pelleted as before. After two additional washes at 4° the cells were resuspended in 70 µl of saline and sealed into glass capillary tubes. At this time, as determined by their capacity to exclude trypan blue dye, the viability of cultured cells or macrophages either shaken or jetted off of their substratum exceeded 95%. Tumor macrophages scraped from plastic wells ranged in viability from 70 to 85%, but tightly adherent, thioglycollate-elicited peritoneal macrophages treated similarly were rarely more than 50% viable. Because of the relatively low viability of the latter cell preparation, in some experiments thioglycollate macrophages were cultured and analyzed while still adhering to the inner surfaces of microslides. Under these conditions >95% of the thioglycollate-elicited, adherent macrophages remained viable throughout the washing, labeling and spectrometric examination processes. Electron spin resonance spectra were recorded with a Varian (Palo Alto, Calif.) model E-104 spectrometer equipped with a temperature controller and interfaced with a Nicolet (Madison, Wisc.) model 535 signal averager. Sample temperatures were measured as described earlier (9). Instrument settings: modulation amplitute, 2.0 G; gain, 5×10^3; microwave power, 10 mW; temperature 21°C.

macrophage membranes, i.e. a rapid signal decay at 37°C that could be slowed by addition of ferricyanide. (b) In our studies labeling was performed at 4°C, a temperature at which "flip-flop" rates of lipid labels across membranes are extremely small (12).

Changes in distance between the outer hyperfine maxima ($2T_{\parallel}$ value) reflect alterations in the anisotropic rotation of the spin-carrying nitroxyl group. The $2T_{\parallel}$

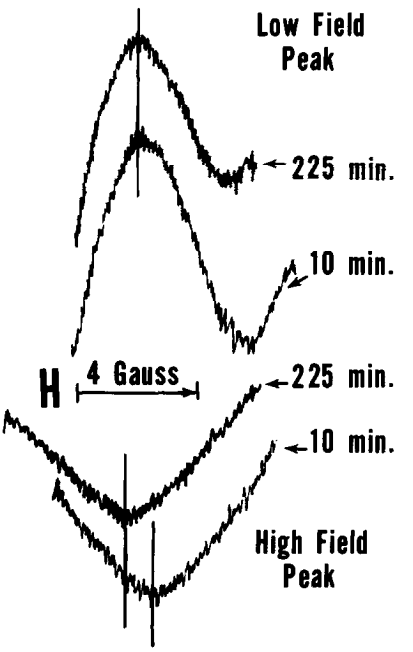

Figure 2. Effect of lipopolysaccharide (LPS) on $2T_{II}$ value of 5-doxyl stearate incorporated into membranes of primed macrophages. The low and high field extrema were recorded at different mid-field settings (difference: 50G) as described by Gordon and Sauerheber (13). Lower curve of each pair was recorded 10 min after LPS addition and upper curve 225 min after LPS addition. Instrument settings: modulation amplitude, 6.3 G; gain, 2.5×10^4; microwave power, 10 mW; temperature, 37°C.

value can therefore be used to assess the freedom of motion of this group and, thus, to characterize the rigidity of the lipid environment in which it is located (10). When incorporated into the plasma membrane of primed macrophages, 5-doxyl stearate showed a hyperfine splitting value indicative of a moderate viscosity. Incubation of these cells with lipopolysaccharide (100 ng/ml) did not immediately change the $2T_{II}$ value rather the hyperfine splitting value decreased with time, reaching a minimum 3-4 h after the cells were exposed to lipopolysaccharide (Fig. 2). This phenomenon was observed consistently in four separate experiments (Fig. 3 a, b). By 5-6 h after exposure $2T_{II}$ returned to its oritinal value, and no further change was detectable up to 16 hours. No membrane perturbation was found when primed macrophages were examined either in the absence of lipopolysaccharide (Fig. 3b) or after they were fixed with low con-

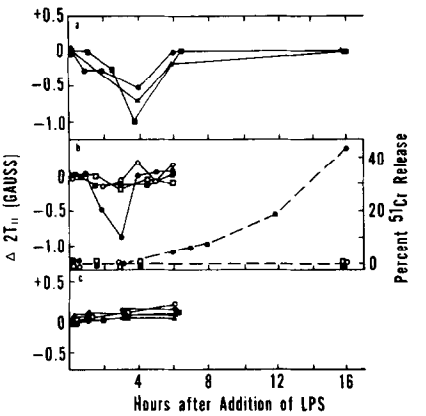

Figure 3. Relationship of membrane perturbation in, and the onset of killing by, lipopolysaccharide-stimulated primed macrophages at 37°C.

(a) Maximal hyperfine splitting values ($2T_{||}$) measured at a given time were substracted from the values recorded at zero times ($\Delta 2T_{||}$) and plotted as a function of time after the addition of lipopolysaccharide (100 ng/ml). Symbols (● , ■ , ▲) refer to individual experiments performed with three different preparations of primed macrophages.

(b) $\Delta 2T_{||}$ values (——) of primed (circles) or thioglycollate induced (squares) macrophages incubated with (closed symbols), or without (open symbols), lipopolysaccharide (100 ng/ml). Also shown is the extent to which P815 mastocytoma cells were killed by primed (circles) or thioglycollate-induced (squares) macrophages incubated with (closed symbols), or without (open symbols), lipopolysaccharide (100 ng/ml). Killing was measured by assaying ^{51}Cr release (— —) from pre-labeled target cells.

(c) $\Delta 2T_{||}$ values of P388D1 cells (circles), MSC tumor cells (triangles), or primed macrophages fixed with 0.025% glutaraldehyde (squares) and incubated with (closed symbols), or without (open symbols), lipopolysaccharide (100 ng/ml).

centrations of glutaraldehyde before exposing them to lipopolysaccharide (Fig. 3c). Similarly, no evidence for the membrane change could be detected after cells of either the continuous macrophage line P388 D1 (Fig. 3c), peritoneal macrophages elicited by thioglycollate broth injection (Fig. 3b), or MSC tumor cells (Fig. 3c) were incubated with lipopolysaccharide. The findings thus clearly demonstrate that minute amounts of lipopolysaccharide cause a transient perturbation in the membranes of primed macrophages and, at least of the cell types studied, that the change was

unique to primed macrophages. The date also show that perturbation of the plasma membranes of these mononuclear phagocytes preceded, by approximately 2 h, the earliest detectable release of ^{51}Cr from target cells, but do not indicate whether or not these phenomena are related.

DISCUSSION

The degree of perturbation measured in the membranes of primed macrophages stimulated by lipopolysaccharide equaled or exceeded that described in other systems in which membrane changes have been associated with alteration in the character of a cell. For example, prostaglandin-dependent deformability of erythrocytes increased or decreased with either a rise or fall, respectively, in membrane fluidity, as Kury and McConnell (14) demonstrated by the spin label technique. These authors suggested that the changes they observed were directly attributable to enzymatic phosphorylation of membrane proteins.

The largest membrane change that we recorded (a 1-to-2% change in the $2T_{||}$ value, which corresponds to a 1.3 to 2.5% change in order parameter) could reflect major shifts in membrane composition. Gaffney (15) has provided evidence, for instance, that (i) a 10% change in the amount of unsaturated fatty acid chains, (ii) a 5% variation in the mole fraction of cholesterol in membrane lipids, or (iii) a change of 20-30% in the fraction of proteins that interact with the membrane is required to cause a spectroscopic change of the magnitude we recorded. Because of its transient nature, however, we consider it unlikely that major alterations in membrane composition were responsible for the perturbation produced in the plasma membranes of primed macrophages by lipopolysaccharide. Instead we believe that a lateral redistribution of membrane constituents is more likely the cause. Such phase separations are easily detectable in other biological membranes by the spin label technique (16), and often are elicited by changes in the intracellular concentration or cellular binding of Ca^{++} (17,18).

The relationship between the observed membrane change and the subsequent onset of killing is unknown. It is conceivable that the perturbation represents transmembrane processing of the signal that initiates killing. At this point, however, we can do no more than relate the two events temporally, in spite of the fact that in the studies reported here, neither the change in the membrane nor the onset of killing developed independent of the other. Our current efforts are focused on determining whether or not the two phenomena are linked.

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