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ACTIVATED MACROPHAGES KILL TUMOR CELLS INDEPENDENT OF MEMBRANE FLUIDITY

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SUMMARY: By using electron spin resonance techniques and 5-doxyl stearate spin labels, we have measured the apparent membrane fluidities of several kinds of macrophages. The tested cells included non-cytolytic thioglycollate-elicited peritoneal macrophages and either cytolytic or non-cytolytic macrophages isolated from regressing Moloney sarcomas. Our results indicated no characteristic plasma membrane fluidity that distinguishes killer macrophages from their non-killing counterparts. Furthermore, no relationship existed between the generalized plasma membrane fluidities of activated macrophages and the neoplastic target cells that they killed.

Macrophages in a state that, for lack of a clearer definition, is called "activated" can kill tumor cells (1,2). The mechanism involved in the killing process is not known; however, it is non-phagocytic in nature, and contact (or extremely close juxtaposition) between effector and target cells is required (3,4). Apparently, the activated macrophage somehow distinguishes between normal and neoplastic cells and kills only the latter (3-5). Hibbs (5,6) has postulated that such "recognition" is the result of similar fluidities in the plasma membranes of activated macrophages and transformed cells.

We decided to test this hypothesis directly with our recently developed system in which the cytolytic activity of macrophages isolated from spontaneously regressing Moloney sarcomas (regressor macrophages) can be modulated predictably and consistently. Freshly explanted macrophages kill neoplastic cells but after 24 h in vitro lose their cytolytic activity entirely (7). However, minute amounts (pg-to-ng/ml) of bacterial lipopolysaccharide (LPS, endotoxin)

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will trigger the sudden resurgence of high levels of this killing activity (8). Thioglycollate-elicited peritoneal macrophages cannot be stimulated to kill by similar concentrations of LPS (8).

Electron spin resonance (ESR) spectroscopy, using fatty acid spin labels, was used to search for possible relationships between cytolytic activity and plasma membrane fluidity. This technique has been employed recently to show that the plasma membrane of non-cytotoxic macrophages isolated from tumors is transiently perturbed when these cells are triggered to kill neoplastic target cells by exposure to minute amounts of LPS (9). The results we report here show that: (i) There is no characteristic plasma membrane fluidity which distinguishes activated macrophages, and (ii) No relationship exists between the plasma membrane fluidities of activated macrophages and the neoplastic cells that they kill.

MATERIALS AND METHODS

Mice and Tumor Induction: BALB/c AnCr male mice aged 6-10 weeks were used. Moloney sarcomas, which previously have been shown to regress spontaneously (10), were induced by the intramuscular (gastrocnemius) injection of 5×10^3 cultured MSC rhabdomyosarcoma cells eleven days earlier (11).

Tumor Disaggregation and Isolation of Macrophages: Tumors were minced and disaggregated enzymatically using a mixture of trypsin, collagenase, and deoxyribonuclease (12). Macrophages were isolated in monolayers on the bottoms of 16 mm-diameter, flat bottomed, plastic wells, as described (7,8). The plating procedure yielded an adherent population of cells that numbered 1.5×10^6/well, as determined by direct counting of cells washed from wells. Their appearance and ability to phagocytose zymosan particles established that 90 to 95% were macrophages. The other cell type consistently found in these monolayers -- usually $<5\%$ but sometimes up to 10% of the total -- was the MSC cell 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 (H-MEM) containing 30% fetal bovine serum (FBS). Thioglycollate was used to elicit peritoneal macrophages and they were harvested as previously described (8). Monolayers containing $>95\%$ macrophages 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, NJ). Non-adherent cells were removed from these monolayers by repeated, vigorous washings.

LPS and Assay of Macrophage-Mediated Cytolysis: Purified LPS isolated from 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 have been described (8). As before (7,8), the amount of chromium-51 (^{51}Cr) released from prelabeled P815 mastocytoma cells in 16 h was used to indicate macrophage-mediated cytolysis.

Spin Labeling of Plasma Membranes: Adherent cell types were scraped (rubber policeman) or jetted free (Pasteur pipette) from their substratum, washed by centrifugation (5 min, 4°,

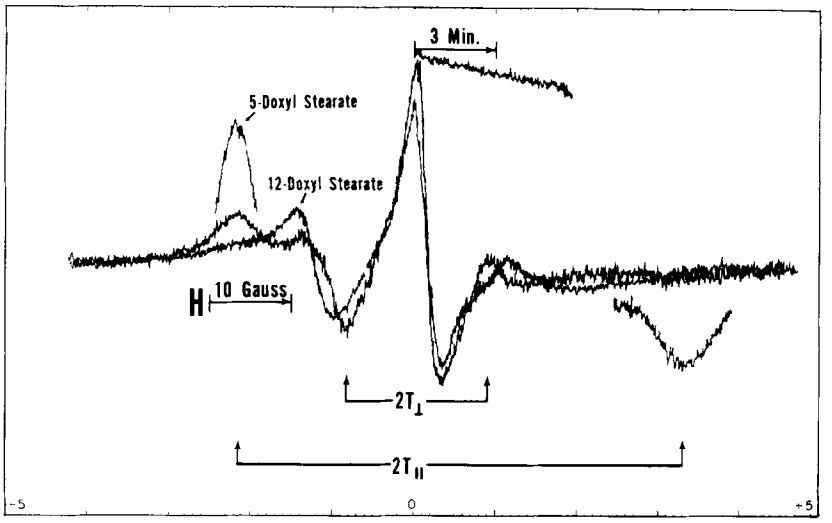

Figure 1. ESR spectra for the two different spin labels in the membranes of macrophages isolated from regressing Moloney sarcomas. The outer peaks are retraced at a higher sensitivity setting to facilitate accurate measurements of $2T_{||}$ values. Rate of decay of the signal at this temperature is indicated by the sloping trace at the top of the figure. Instrument settings: Modulation amplitude, 2.0 G; microwave power, 10 mW; temperature, 21°C.

600 g_{max}) and resuspended in 100 µl of ice cold PBS. The final cell concentration was usually 2×10^7/ml. Spin labels (either 5- or 12-doxyl stearate from SYVA, Palo Alto, CA) were dissolved in ethanol (125 µg/ml). Seven microliters of this solution were added to the chilled cell suspension. After 10 min the cells were diluted to 2 ml with ice cold PBS and pelleted as before. After two additional washes at 4° the cells were resuspended in 70 µl of PBS and sealed into glass capillary tubes. The viability (trypan blue dye exclusion) of cultured cells or macrophages jetted free from their substratum exceeded 95%. Tumor macrophages scraped from plastic wells were 70 to 85% viable, but tightly adherent, thioglycollate-elicited peritoneal macrophages treated similarly were rarely more than 50% viable. Because of the latter's relatively low viability, in some experiments thioglycollate-induced macrophages were cultured and analyzed in situ on microslides. Under these conditions >95% of the thioglycollate-elicited, adherent macrophages remained viable throughout the labeling and examination processes.

ESR spectra were recorded with a Varian (Palo Alto, CA) model E-104 spectrometer equipped with a temperature controller and interfaced with a Nicolet (Madison, WI) model 535 signal averager. Sample temperatures were measured as described earlier (13). Hyperfine splitting parameters were determined either from the computer averaged and expanded spectra or by the method of Gordon and Sauerheber (14). For further details see Figure 2 of reference (9).

RESULTS AND DISCUSSION

Figure 1 shows that the spectra for the 5-doxyl and 12-doxyl stearate were different. This difference denotes that the labels were located within a flexibility gradient in a membrane

TABLE I

HYPERFINE SPLITTING ($2T_{||}$) VALUES OF 5-DOXYL STEARATE IN MEMBRANES OF MOUSE MACROPHAGES (MØ) AND NEOPLASTIC CELL LINES

CELLS	$2T_{||}$ (GAUSS)*	
	21°C	37°C
NONCYTOLYTIC† MACROPHAGE:		
1. Thioglycollate-elicited peritoneal MØ	55.2 ± 0.3	51.9 ± 0.5
2. Cultured MØ (isolated from regressing Moloney sarcoma and held 24 h in culture)	54.8 ± 0.4	51.0 ± 0.5
CYTOLYTIC† MACROPHAGE:		
1. Freshly explanted from regressing Moloney sarcoma	55.2 ± 0.3	51.3 ± <0.1
2. Cultured MØ stimulated with LPS (100 ng/ml, 16 h)	55.0 ± 0.3	51.5 ± 0.5
NEOPLASTIC TARGET CELL LINES:		
1. P815 mastocytoma	56.0	51.8
2. MSC rhabdomyosarcoma	53.8 ± 0.2	50.8 ± 0.2

* Mean ± standard deviation of four or more separate determinations. Values without standard deviations are of single determinations.

† Cytolytic capacity was determined by ^{51}Cr release from target cells as described under Methods. Release of ^{51}Cr, using P815 mastocytoma and MSC rhabdomyosarcoma cells as targets in a 16 h assay was in the range of 32 to 50%.

rather than merely adsorbed to cell surfaces (15). Also shown in Figure 1 is the rate at which the spin labels were reduced by the cells. As we have described (9), membrane impermeant ferricyanide ions can reoxidize almost all labels. In addition, the impermeant LPS produces a membrane perturbation that can be recorded by such labels. These two facts make it very likely that the fatty acid spin labels are confined within the plasma membrane.

Changes in distance between the outer hyperfine maxima ($2T_{||}$ value) reflect alterations in the anisotropic rotation of the spin-carrying nitroxyl group. Therefore, the $2T_{||}$ value can be used to assess this group's freedom of motion and, thus, to characterize the rigidity of the lipid environment in which it is located (15). When incorporated into the plasma membranes of macrophages, 5-doxyl stearate showed $2T_{||}$ values indicative of a moderate viscosity.

Populations of macrophages that differed in their capacity to kill neoplastic cells were measured at 21° and 37° in terms of $2T_{||}$ values (Table I). No important differences were found between the plasma membranes of these various macrophage populations, in spite of the fact that they differed functionally from one another. Also shown in Table I are $2T_{||}$ values obtained from the analyses of two different neoplastic target cell lines. As expected (7), these cells were susceptible to killing by activated macrophages (Table I). However, as shown in Table I, no clear similarity was found in the fluidities of their plasma membranes as measured by 5-doxyl stearate and neither of these cell types had $2T_{||}$ values that resembled those of cytolytic macrophages.

Thus, the selective killing of tumor cells by activated macrophages cannot be explained on the basis of generalized similarities between the membrane fluidities of the effector and target cells. These results argue against the theory that Hibbs (6) has put forth to explain the selectivity of the killing process and should serve to shift attention to other mechanisms that may be responsible for macrophage-mediated killing of tumor cells.

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