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SPECIFIC ANTIBODY-DEPENDENT ACTIVATION OF NEUTROPHILS BY LIPOSOMES CONTAINING SPIN-LABEL LIPID HAPTENS

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SUMMARY: We have found that superoxide production by human neutrophils can be stimulated by liposomal membranes containing nitroxide spin-labeled lipids in the presence of specific rabbit antibodies directed against the nitroxide group. The extent of superoxide production was found to depend strongly on lipid composition under conditions where the concentration of antibodies and number of exposed haptens were maintained constant. The dependence of neutrophil activation on the physical properties of the liposomal lipid membrane appears to be similar to the dependence of complement depletion on these physical properties.

INTRODUCTION: There is considerable interest in the use of synthetic membranes to study the elementary molecular events involved in immune recognition and triggering (1-9). In such studies systematic variations in membrane physical properties and chemical composition can provide significant clues as to the kinetic and structural factors that are critical for recognition and triggering. It is equally true that systematic comparative studies of different afferent and efferent elements of the immune systems, with respect to their interactions with these synthetic membranes, can also provide clues regarding these structural and kinetic factors. In previous work we

Abbreviations: Cl and Clq are the components of complement according to WHO Recommendations (1968). DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; egg PC, egg phosphatidylcholine; chol, cholesterol; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid.

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have used lipid vesicles and liposomes containing haptens as the synthetic membranes, and have studied their interactions with complement, C1[1], C1q, and macrophages (4-6, 10-13). Related studies have been carried out in other laboratories (1, 3, 7, 8). In the present work we describe a preliminary investigation in which the effector component is the human neutrophil, and the target membranes are the same spin-label nitroxide hapten-sensitized liposomes used in previous studies. The neutrophil constitutes about 50% of all blood leukocytes. Its primary function is phagocytosis and killing of microbial organisms (14). Specific antibody-dependent recognition of targets via Fc receptors on the neutrophil (15) triggers rapid superoxide anion ($O_2^{\cdot-}$) production. This burst of $O_2^{\cdot-}$ production appears to be important for microbial killing (16) and also provides us with a convenient assay for specific antibody-dependent recognition and triggering using synthetic membranes.

MATERIALS AND METHODS: Neutrophils were isolated from venous blood collected from normal human volunteers. Fibrin and platelets were removed by gentle swirling of the blood with 3 mm diameter solid glass beads (~3 beads/ml) for 10 min. Blood was diluted with 4 volumes of 0.85% NaCl, and then granulocytes and erythrocytes were separated from mononuclear leukocytes by centrifugation through Ficoll-Hypaque as described by Boyum (17). The pellet, containing erythrocytes and granulocytes, was mixed with 8 volumes of plasma gel (30 g gelatin, 7 g NaCl, and 2.0 g $CaCl_2 \cdot 2H_2O$ per liter) and allowed to sediment at 24° for 30 min. The granulocyte-rich supernatant was centrifuged at 300×g for 15 min, the pellet collected, and the 1×g sedimentation procedure repeated at 5% of the original plasma gel volume. These leukocyte preparations contained 90-95% neutrophils with 1-10 erythrocytes per leukocyte. The cells were washed twice in Ca^{++}- and Mg^{++}-free Earle's balanced salt solution buffered with 25 mm HEPES. Prior to use, the neutrophils were suspended at 5×10^6 per ml in cell buffer (2.0 mM $CaCl_2$, 1.5 mM $MgCl_2$, 5.4 mM KCl, 1 mM $Na_2H_2PO_4$, 5.6 mM glucose, and 120 mM NaCl. Liposomes were prepared essentially by the method of Schwartz and McConnell (18). Briefly, the required amounts of lipid and lipid hapten were dissolved in methanol and evaporated to dryness in a 5-ml round bottom flask. Cell buffer was added to the flasks and the lipid was allowed to

[1] Studies on C1 activation have been carried out by A. F. Esser, R. M. Bartholomew, J. W. Parce and H. M. McConnell, and have been submitted for publication.

hydrate for 15 min at a temperature greater than 15° above the chain melting temperature of each particular phospholipid. The lipids were then dispersed by vigorous vortexing. All liposomal suspensions contained 1 mM total lipid, 1% of which was the nitroxide spin-label hapten.

Preparation of antinitroxide IgG was carried out as follows. After heat inactivation of complement (56°, 30 min), immune sera from rabbits immunized with spin-labeled keyhole limpet hemocyanin were titrated with enough non-labeled keyhole limpet hemocyanin to form a maximum immune precipitate. The precipitate, which was allowed to form for 1 hr at 37° and for an additional 4 hr at 4°, was then centrifuged for 10 min at 10,000 rpm in a Lourdes 9 RA rotor (5). The supernatant was adjusted to pH 7.5 and passed over a protein-A sepharose column (Pharmacia). After washing all unbound protein off the column with .01 M phosphate buffer pH 7.5 containing 0.15 M NaCl, the IgG fraction was eluted with 0.1 M acetate buffer pH 4.0. This IgG fraction was adjusted to pH 7 by the addition of 1 M K_2HPO_4. By measuring the binding of free spin labels to this preparation it was determined to contain approximately 7 μM specific antinitroxide IgG. This stock preparation was diluted 1:10 with cell buffer just before each experiment.

For cell stimulation studies, 100 μℓ of diluted antibody and 100 μℓ of liposomal suspension were mixed in a 10 × 75 mm glass test tube. After 20 min preincubation at 23°, 100 μℓ of 25 mM cytochrome c (horse heart Type III, Sigma Chemical Company, St. Louis, MO) in cell buffer was added. To activate $O_2^{\cdot-}$ production, 200 μℓ of the neutrophil suspension was added, mixed, and within 1 min placed in a 37° water bath shaker (170 cycles/min). The reaction was stopped after 15 min by placing the tubes in ice water. The release of $O_2^{\cdot-}$ was measured by the amount of cytochrome c reduced during the 37° incubation (19, 20). Cells and liposomes were sedimented at 8,000×g at 4° for 10 min in 1.5 ml plastic centrifuge tubes and the supernatant quickly removed. Cytochrome c reduction was calculated from the increase in absorbance at 550 nM using an absorbance coefficient of 2.11×10^4 M^{-1} cm^{-1} (21).

The following controls were carried out: essentially zero activation was obtained in the absence of (1) hapten, or (2) antibody, or (3) liposomes at all antibody concentrations. Also, antibodies at the highest concentration in the presence of water soluble hapten showed no activation, under conditions where 80% of the hapten was bound. Since all erythrocytes were not removed from neutrophil preparations, periodic checks were made to insure that the optical density at 550 nm was not due to hemolysis. The optical density at 541.75 and 556.5 nm (isobestic points for the reduced and oxidized cytochrome c spectra (22)) showed no increases under conditions of activation. Stimulations reported in the present paper are equal to or less than 40% of the maximal stimulation caused by 10 μg/ml phorbol myristate acetate (12-0-tetradecanoyl phorbol-13-acetate, Consolidated Midland Corp., Brewster, NY). For comparable conditions, neutrophils obtained from three different donors gave equivalent results.

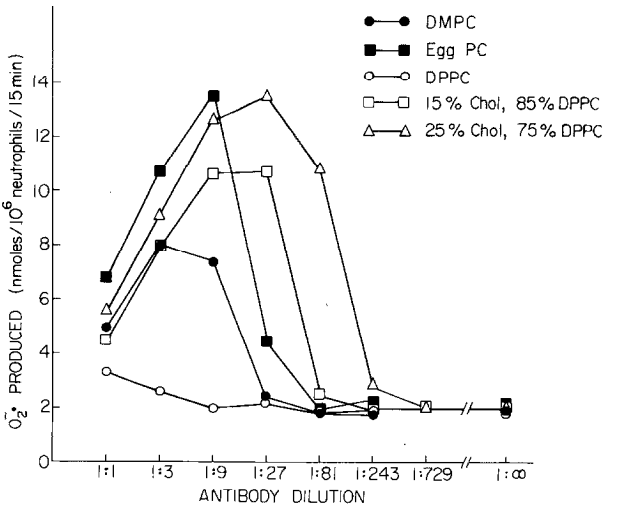

Figure 1. Nanomoles of superoxide released per 10^6 neutrophils as a function of specific rabbit antinitroxide serial dilution. In the reaction mixture the 1:1 specific antibody concentration is estimated to be of the order of 10^{-7} moles/liter. The neutrophil concentration is 2×10^9 cells/liter and the liposomes are present at a concentration of 2×10^{-4} moles/liter phospholipid. Hapten was present as 1 mole % of total lipid (phospholipid plus cholesterol). Since ca. 5% of the total hapten on these liposomes is on the outermost membrane surface (18), the total exposed hapten concentration is of the order of 10^{-7} moles/liter.

RESULTS AND DISCUSSION: The results presented in Fig. 1 clearly demonstrate a specific antibody-dependent stimulation of neutrophils by liposomes containing 1 mole % nitroxide spin label lipid hapten. The stimulation is biphasic in specific antibody concentration in all cases. Even in the case of liposomes composed of dipalmitoylphosphatidylcholine (DPPC), separate experiments using a longer preincubation of antibody and liposomes showed the biphasic character of the stimulation (data not shown). Biphasic responses are also seen in complement depletion, C1q binding, and C1 activation[1] using similar preparations of hapten-sensitized liposomes and specific antibodies against the nitroxide group (6, 9-11).

The reduction of Clq binding, complement depletion, and neutrophil activation at high antibody concentrations is evidently due to the fact that under these conditions antibodies bind to the membrane with only one Fab binding site, and thus do not undergo a conformational change to an activated state. This is consistent with the observation that neither complement nor neutrophils are activated by specific antibodies bound to free hapten in aqueous solution.

The results seen in Fig. 1 are also similar to measurements of complement depletion and C1 activation in that the "fluid" membranes, dimyristoylphosphatidylcholine (DMPC) and egg phosphatidylcholine (egg PC) at 37°, showed a significantly higher degree of activation of the neutrophils than did the "solid" membrane (DPPC at 37°). Data not given in Fig. 1 also showed that hapten-sensitized diphytanylphosphatidylcholine membranes--also fluid at 37°--gave a biphasic activation of neutrophils similar to DMPC and egg PC at 37°.

As is evident in Fig. 1, inclusion of 15 or 25 mole % cholesterol in DPPC membranes greatly enhances the activation of neutrophils relative to DPPC membranes alone, again a result observed previously with complement depletion. There is clearly no simple correlation between the cholesterol-mediated enhancement of neutrophil activation and the recently reported cholesterol-mediated enhancement of lipid lateral diffusion in DPPC membranes (23) at temperatures below the chain melting temperature of the phospholipid (42°).

We wish to emphasize that the hapten-sensitized liposomes used in the present study are not merely another type of antigen, or a multiplicity of haptens coupled to a support. First, these preparations mimic in many respects the plasma membranes of cells.

Secondly, extensive studies of the paramagnetic resonance spectra of the hapten groups show that lipid haptens diffuse freely in the fluid membranes, and are not detectably clustered in any of the membranes used in the present study. Even in solid phase membranes, as judged from studies of the motion of simple fluorescent-labeled lipids (24), it is virtually certain that the lipid haptens diffuse slowly but probably at a chemically significant rate. Finally, freeze-etch studies have shown that hapten-bound antibodies in these liposomes do not spontaneously aggregate to a significant degree (5). Thus, if the "unit signal" for neutrophil activation involves a clustering of antibodies, the driving force for clustering of antibodies (plus probably Fc receptors) must be provided by the cell.

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