Triggering of the Macrophage and Neutrophil Respiratory Burst by Antibody Bound to a Spin-Label Phospholipid Hapten in Model Lipid Bilayer Membranes†

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ABSTRACT: The specific antibody-dependent stimulation of the respiratory burst (cyanide-insensitive oxygen consumption, 1-*C*-glucose oxidation) of RAW264 macrophage cell line by haptenated lipid vesicles depends strongly on the physical properties of the lipid membrane, as well as the surface density of antibodies on the vesicles. Lipid membranes that are "solid" at 37 °C (dipalmitoylphosphatidylcholine, DPPC) are much more effective, per vesicle bound, than are "fluid" membranes (dimyristoylphosphatidylcholine, DMPC). Vesicle membranes that have both fluid and solid regions (DPPC containing <20 mol % cholesterol) show both enhanced binding rates (due to the fluid regions) and enhanced respiratory rates (due to the solid regions). In contrast to these results, the specific antibody-dependent respiratory burst of neutrophils due to haptenated vesicles parallels the antibody-dependent vesicle binding and shows no significant difference between fluid and solid target membranes.

At present a variety of studies have been reported on the interactions of various components of the immune system with model membranes (lipid bilayers forming vesicles or liposomes) containing specific haptens or antigens (Esser et al., 1979; Finberg et al., 1978; Geiger & Schreiber, 1979; Hale et al., 1980; Kinsky & Nicolotti, 1977; Hafeman et al., 1979; Henkart & Blumenthal, 1975; Henry et al., 1978; Hollander et al., 1979; Lewis & McConnell, 1978; Loh et al., 1979; McConnell, 1978; Parce et al., 1978, 1980). Some of these studies have described a dependence of immune response on the physical–chemical properties of the host bilayer membranes (Esser et al., 1979; Hafeman et al., 1979; Lewis & McConnell, 1978; Parce et al., 1980; McConnell, 1978). For example, the activation of the first component of complement C_1 and complement depletion are enhanced in "fluid" DMPC[1] membranes relative to "solid" DPPC membranes (Esser et al., 1979; Parce et al., 1980). Likewise the inclusion of cholesterol in otherwise solid DPPC membranes leads to an enhancement in complement depletion (Humphries & McConnell, 1975; Brûlet & McConnell, 1977). We refer to bilayer membranes as "fluid" or "solid", depending on whether the lateral diffusion coefficients of lipid haptens, and antibodies bound to these haptens, are $\geq 10^{-8}$ cm^2/s or $\leq 10^{-10}$ cm^2/s (Smith et al., 1979). In the accompanying paper (Lewis et al., 1980) we show that the kinetics of specific antibody-dependent binding of haptenated lipid vesicles is likewise enhanced in fluid DMPC vesicles compared to solid DPPC vesicles and is enhanced when cholesterol is included in DPPC vesicles. In the present paper we report an unexpected reversal of these correlations, in that solid haptenated DPPC vesicles are found to be far more effective than fluid DMPC vesicles (per vesicle bound) in stimulating the respiratory burst in the macrophage cell line RAW264. Likewise, inclusion of cholesterol in DPPC decreases this respiratory burst. Thus, markedly different membrane molecular properties are involved in recognition (binding) and in cell triggering.

It has been established that macrophage cell lines such as RAW264 can lyse tumor targets and red blood cells (Ralph & Nakoinz, 1977). In spite of extensive efforts, we have been unable to observe specific antibody-dependent lysis of lipid hapten containing large liposomes composed of distearoylphosphatidylcholine and cholesterol (50:50 mol %). In addition, giant vesicles (40–100 μm in diameter) composed of either egg phosphatidylcholine or mixtures of DMPC, DPPC, and cholesterol (25, 45, 30 mol %, respectively) showed specific antibody-dependent binding but no detectable lysis (T. Lewis, unpublished experiments). [On the other hand, vesicles containing bovine eye muscle membrane protein to which thyroglobulin–antithyroglobulin complexes were bound can be lysed by RAW264 macrophages (T. Lewis and Q. Mehdi, unpublished experiments).] Thus, we neglect any consideration of external lysis in the present paper because this process does not appear to compete with phagocytosis or the triggering of the respiratory burst.

Materials and Methods

Cells, Vesicles, Lipid Hapten, Antibodies, Vesicle Binding Assay, and Microscopy. All of these materials and methods are the same as those described in the accompanying paper (Lewis et al., 1980) except for the neutrophils, which were prepared as described elsewhere (Hafeman et al., 1979). For a reference to studies of antibody binding to DMPC and DPPC vesicles, see the first paragraph of Results of the accompanying paper.

1-C-Glucose Oxidation. Glucose oxidation and vesicle binding were measured under similar incubation conditions. Polypropylene vials (1.2 × 5.0 cm, E & K Scientific Products, Inc., Saratoga, CA) were used in the glucose oxidation assay. A special cell buffer [2.0 mM $CaCl_2$, 1.5 mM $MgCl_2$, 5.4 mM KCl, 1 mM Na_2HPO_4, 5.6 mM glucose, 120 mM NaCl, and 0.2% bovine serum albumin (Calbiochem, fatty acid poor), pH 7.4] was used in the binding, glucose oxidation, oxygen consumption, and superoxide release assays. Vesicles (100 μL, 0.020 or 0.025 μmol of total lipid suspended in 0.15 M NaCl) and antibody (previously diluted into 100 μL of cell buffer) were carefully pipetted into each polypropylene vial as phys-

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[1] Abbreviations used: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced NADP.

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ically separate drops, so that vesicles and antibody did not mix. Each vial also contained 0.24 μCi of [1-^{14}C]glucose (50–60 μCi/μmol, Amersham Corporation, Arlington Heights, IL) in 12 μL of 0.15 M NaCl. From 15 to 30 min prior to incubation with macrophages, antibody and vesicles were thoroughly mixed by swirling the vials rapidly. Macrophages (1×10^6) were added in cell buffer to give a total incubation volume of 0.5 mL per vial. To collect respired CO_2, we placed a half-circle of Whatman GF/A 2.4 cm diameter filter paper saturated with 50 μL of 20% KOH into retaining wells previously drilled into the bottoms of rubber stoppers. The rubber stoppers were then inverted and used to cap each vial.

The mixtures were incubated at 37 °C in a gyrotary water-bath shaker (Model G76, New Brunswick Scientific Co., New Brunswick, NJ) at 170 cycles/min. [In one experiment the polypropylene vials were replaced by polystyrene tissue culture wells (2.0-cm^2 area per well, Linbro Division, Flow Laboratories, Inc., Hamden, CT). The wells were capped by rubber stoppers as described above and placed in a 37 °C tissue culture incubator for 1 h without stirring. All other procedures of the assay were identical with those used with polypropylene vials, as described below.] After 1 h the vials were chilled in ice water. HCl (2.0 N, 50 μL) was added to each vial which was quickly recapped. Thirty minutes later the stoppers were removed and filter papers dried. Radioactivity was determined by liquid scintillation counting with 5.0 mL of toluene-based fluor (4.6 g/L preblend 2a70, RPI Corp., Elk Grove, IL). The counting efficiency was 80%.

Oxygen Consumption. Oxygen consumption was measured with a Gilson Model K-ICRP-O oxygraph and Clark electrode with a 2.0-mL stirred reaction vessel. The buffer system was the same as for glucose oxidation and binding studies except that 2.0 mM KCN was added to inhibit mitochondrial respiration. The concentrations of cells and vesicles were increased to 1×10^7 macrophages and 0.25 μmol of lipid ("large" vesicle size fractions) per 2.0-mL reaction volume. Oxygen concentration in air-saturated buffer at 37 °C was taken as 199 μM (Chappell, 1964), and zero oxygen was determined by the addition of sodium dithionite.

Superoxide Release. All cells and reagents were diluted into cell buffer prior to use. Diluted antibody (100 μL) and vesicle suspension (100 μL, 0.025 μmol of lipid) were mixed in 10 × 75 mm glass test tubes. After a 20-min preincubation at 23 °C, 100 μL of 0.25 mM cytochrome *c* (horse heart, type III, Sigma Chemical Co., St. Louis, MO) was added. For activation of O_2^- production, 200 μL of neutrophil suspension (10^6 cells) was added, mixed, and within 1 min placed in a 37 °C water-bath shaker (170 cycles/min). The reaction was stopped 15 min later by placing the tubes in ice water. The release of O_2^- was measured by the amount of cytochrome *c* reduced during the 37 °C incubation (Babior et al., 1973; Goldstein et al., 1975). Cells and vesicles were sedimented at 7000*g* at 4 °C for 10 min in 1.0-mL polystyrene centrifuge tubes and the supernatant was quickly removed. Cytochrome *c* reduction was calculated from the increase in absorbance at 550 nm with an absorbance coefficient of 2.11×10^4 M^{-1} cm^{-1} (Van Gelder & Slater, 1962).

Superoxide release was measured only with neutrophils since the RAW264 cell line does not release O_2^- upon stimulation. This result is obtained by using either antibody-coated vesicles or phorbol myristate acetate (12-*O*-tetradecanoylphorbol 13-acetate; Consolidated Midland Corp., Brewster, NY) with each cell type.

Results

As shown previously (Lewis et al., 1980) macrophages, during 1-h incubations, do not bind either haptenated DPPC or DMPC vesicles in the absence of specific antibody. Increases in the surface density of vesicle-bound, antinitroxide antibodies result in vesicle binding after a critical antibody surface density is exceeded. Vesicles of DPPC, for which the lateral diffusion of haptens is low, require a greater antibody density to bind macrophages than do DMPC vesicles, where lateral diffusion of haptens is high. This phenomenon can be interpreted in terms of a requirement for diffusion of a minimum number of vesicle-bound antibodies to macrophage Fc receptors during effective macrophage–vesicle collisions (Lewis et al., 1980).

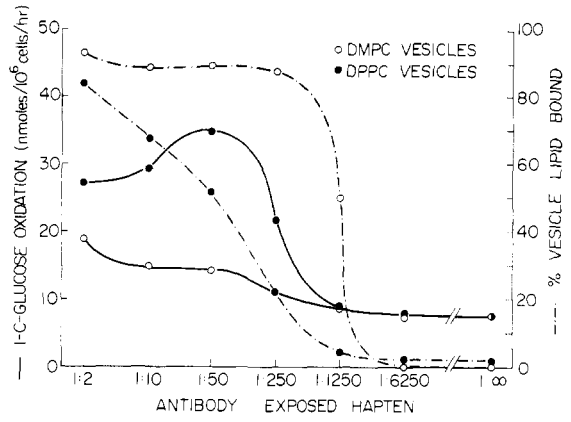

FIGURE 1: Oxidation of exogenous 1-*C*-glucose by the RAW264 macrophage cells and binding of DMPC or DPPC vesicles with 1 mol % nitroxide spin-label phospholipid hapten and various concentrations of antinitroxide antibody. The total amount of vesicle lipid was 0.025 μmol (50 μM), and the "large"-size fractions of the vesicles were used. Incubation was 1 h at 37 °C. Antibody was supplied as affinity-purified antinitroxide IgG; exposed hapten was 1.5×10^{-7} M. Points show the mean of duplicate determinations. Resting macrophages, in the absence of vesicles, oxidized 8.2 ± 0.4 and 8.3 ± 0.6 nmol (10^6 cells h)$^{-1}$ with either 0 or the highest (1:2) concentration of antibody, respectively.

Figure 1 shows that half-maximal binding of DMPC vesicles is observed when the antibody/exposed hapten ratio is 1:1250 (about 13 specific antibody molecules per μm^2 or 42 antibody molecules per 1 μm diameter vesicle). At an antibody/exposed hapten ratio of 1:250, and at higher antibody surface densities, vesicle binding approaches a maximum value of ~90% of total vesicle lipid during a 1-h incubation. Oxidation of 1-*C*-glucose gradually begins to increase only at the high antibody surface densities, reaching a plateau of twice the resting rate at antibody/exposed hapten ratios of 1:50 and 1:10. At the 1:2 saturating density of antibody, there is a further increase to 2.5 times the resting level of 1-*C*-glucose oxidation. Control studies of macrophages incubated with high concentrations of antibody show no metabolic activation in the absence of haptenated lipid vesicles.

Measurement of 1-*C*-glucose oxidation at the 1:1250 antibody/exposed hapten ratio, which yields 50% maximal binding, shows only insignificant activation of the respiratory burst. Even at the 1:250 antibody/exposed hapten ratio where binding is maximal, 1-*C*-glucose oxidation is only one-half of the plateau value. These results demonstrate that phagocytosis of antibody-coated particles does not lead to obligatory or proportional stimulation of the respiratory burst. This conclusion is confirmed by an experiment (not shown) where the binding of vesicles to macrophages was measured in 10 × 75 mm glass tubes with minimal stirring and a corresponding measurement of 1-*C*-glucose oxidation was performed in polystyrene tissue culture wells with no stirring. Under these conditions, less antibody is required for binding either DPPC

or DMPC vesicles. Appreciable binding of DMPC vesicles was observed at the 1:6250 antibody/exposed hapten ratio and was optimal at 1:1250. Nonetheless, there was no stimulation of 1-*C*-glucose oxidation until the antibody/exposed hapten ratio is 1:250 or greater.

Binding of DPPC vesicles requires a 25-fold higher antibody/exposed hapten ratio for half-maximal binding compared to DMPC vesicles. However, under all conditions where antibody-dependent binding of DPPC vesicles can be detected, 1-*C*-glucose oxidation is always greater than for macrophages triggered by antibodies on DMPC vesicles at the same antibody/exposed hapten ratio. Respiratory activation by DPPC vesicles is optimal at the 1:50 antibody/exposed hapten ratio where 1-*C*-glucose oxidation is 5 times the resting level. A further increase in the density of bound antibody resulted in a slight decrease in 1-*C*-glucose oxidation to 4 times the resting level. This effect, the inability of DMPC vesicles to trigger the respiratory burst upon phagocytosis, and the greater stimulation by DPPC vesicles were all highly reproducible in three additional experiments (not shown).

The marked differences in activation caused by DMPC and DPPC vesicles cannot be attributed to differential antibody binding to lipid hapten. Binding of the specific antinitroxide antibody to liposomes with 1% hapten has been measured for both DMPC and DPPC, for antibody/exposed hapten ratios in the 1:1 to 1:81 range. At high antibody/exposed hapten ratios, 1:1, the binding to DMPC and DPPC liposomes was found to be approximately 55 and 30%, respectively. At lower antibody/exposed hapten ratios (1:81), the percent of antibodies bound to the liposomes was larger (~70%) and the difference between DMPC and DPPC appears to be less (Humphries & McConnell, 1980). The differences between DMPC and DPPC vesicles also could not be attributed to gross aggregation of the more fluid DMPC vesicles, nor could they be attributed to an inability of DMPC vesicles to be phagocytized by macrophages. Fluorescence and interference contrast microscopy of vesicles and macrophages after a 1-h incubation revealed phagocytosis of most, if not all, bound vesicles (Figure 2). Both DPPC and DMPC vesicles are apparently phagocytized as single unaggregated particles. It appears, in every case, that once antibody-dependent binding occurred phagocytosis always followed, regardless of vesicle lipid composition or surface density of bound antibodies. Preliminary experiments using a mouse monoclonal IgG antinitroxide antibody gave results quite similar to those reported above for rabbit antibody except that all plots of vesicle and glucose oxidation were displaced to high antibody concentrations by roughly a factor of 125, presumably due to the known lower affinity of this monoclonal antibody. (The monoclonal antibodies were kindly provided by Frank Howard and Dr. John Owicki.)

The 1-*C*-glucose oxidation assay measures the respiratory burst of macrophages indirectly through the utilization of glucose for NADP reduction to NADPH. The NADPH is subsequently consumed by a membrane-bound NADPH oxidase which reduces molecular oxygen to O_2^-. This enzyme is insensitive to inhibitors of mitochondrial respiration, e.g., cyanide or azide, and is activated by phagocytic and certain pharmacologic stimuli (Cheson et al., 1977; Johnston, 1978). To determine if the Fc receptor mediated metabolic activation observed is due to activation of the NADPH oxidase, we measured cyanide-insensitive oxygen consumption. The resting macrophages consumed 13 nmol of oxygen per 10^7 cells per min, and this consumption was reduced to 3.5 nmol/min in the presence of cyanide (Figure 3). Stimulation of cyanide-insensitive oxygen consumption occurs only when both antibody and vesicles are present. At the 1:25 antibody/exposed hapten ratio, DPPC vesicles enhance oxygen consumption to 19 nmol/min. In contrast, the same amount of antibody on DMPC vesicles results in a consumption of only 7 nmol/min by the macrophages. A mixture of vesicles at the 1:25 antibody/exposed hapten ratio composed of half DMPC vesicles and half DPPC vesicles resulted in a rate of oxygen consumption which was approximately midway between the rate observed for DMPC and the rate observed for DPPC, suggesting that the two kinds of vesicles act independently in stimulating the respiratory burst. The lower antibody/exposed hapten ratio (1:625) fails to stimulate with either type of vesicle. The kinetics of macrophage activation are similar for both DMPC and DPPC vesicles. Oxygen consumption quickly increases to the maximal rate after an ~1-min lag time. This rate slowly declines to the initial level, or below, with an apparent half-time of about 20 min. The addition of cytochalasin B at 2 μg/mL abrogates the antibody-dependent vesicle stimulation of cyanide-insensitive oxygen consumption. Cytochalasin B had the same effect when 2 μg/mL phorbol myristate acetate was used to stimulate oxygen consumption instead of antibodies and vesicles.

After measuring the rate of oxygen uptake for 30 min, the incubation mixtures were stored on ice for subsequent measurement of vesicle binding and visualization of phagocytosis. About 50% of both DPPC and DMPC vesicles bind at a 1:25 antibody/exposed hapten ratio. At the 1:625 antibody/exposed hapten ratio ~10% of the DMPC vesicles are bound. Microscopic observations showed that essentially all bound vesicles were phagocytized and the appearance of fluorescent vesicles and cells after phagocytosis of DMPC or DPPC vesicles was indistinguishable. In the absense of antibody, binding and phagocytosis are <1%.

In an attempt to discover whether the dramatic difference in macrophage response to antibody-coated DMPC and DPPC vesicles was due to a uniformly lower level of macrophage respiratory activation in the case of DMPC or to fewer numbers of macrophages responding at some fixed level, the O_2^- indicator, nitro blue tetrazolium, was used. [In the presence of O_2^-, nitro blue tetrazolium changes from a soluble light yellow compound to a dark blue precipitate which can be seen by bright-field microscopy (Nathan et al., 1969).] Macrophages were stimulated by either DMPC or DPPC vesicles for 30 min in the presence of 0.5 mg/mL nitro blue tetrazolium at the 1:25 antibody/exposed hapten ratio. Ten percent of the macrophages incubated with DMPC vesicles contained large, dark blue grains of nitro blue tetrazolium precipitate, as contrasted with 30% of the macrophages which were stimulated by antibody-coated DPPC vesicles. A closer examination of the macrophages using a combination of bright-field and fluorescence microscopy revealed that >85% of the macrophages contained phagocytized vesicles and that the number of phagocytized vesicles per macrophage seemed to be uniform, irrespective of the lipid composition of the vesicle. However, the distribution of dark nitro blue tetrazolium grains was markedly nonuniform, with respect to distribution among the macrophage populations and within each individual macrophage. Some of the macrophages contained a large number of brightly fluorescent vesicles with no dark nitro blue tetrazolium precipitate, whereas other macrophages contained large amounts of precipitate within structures presumed to be phagolysosomes. In other cases, fluorescent vesicles with no blue precipitate were found to coexist with putative phagolysosomes deeply stained with blue grains. These results suggest

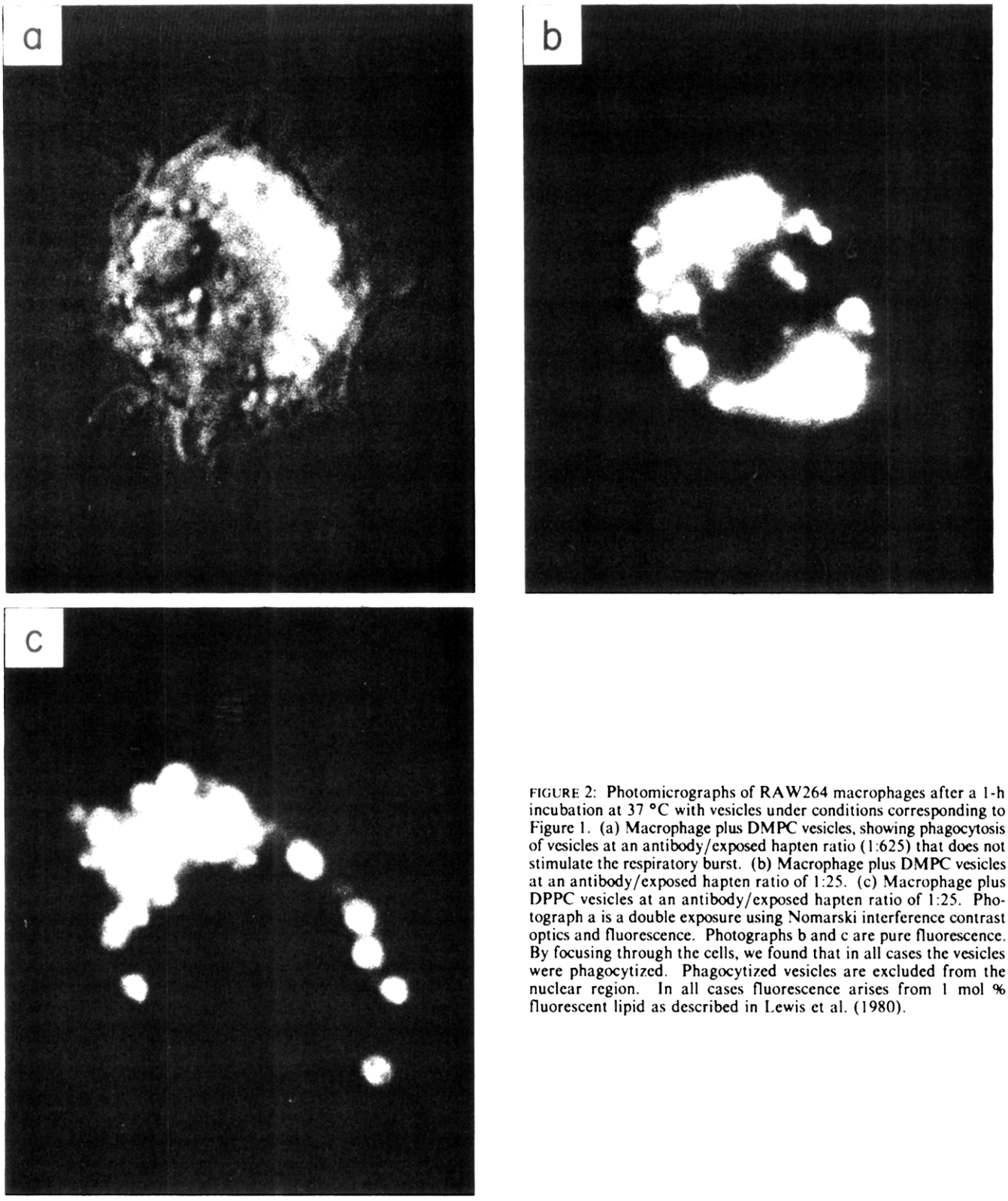

FIGURE 2: Photomicrographs of RAW264 macrophages after a 1-h incubation at 37 °C with vesicles under conditions corresponding to Figure 1. (a) Macrophage plus DMPC vesicles, showing phagocytosis of vesicles at an antibody/exposed hapten ratio (1:625) that does not stimulate the respiratory burst. (b) Macrophage plus DMPC vesicles at an antibody/exposed hapten ratio of 1:25. (c) Macrophage plus DPPC vesicles at an antibody/exposed hapten ratio of 1:25. Photograph a is a double exposure using Nomarski interference contrast optics and fluorescence. Photographs b and c are pure fluorescence. By focusing through the cells, we found that in all cases the vesicles were phagocytized. Phagocytized vesicles are excluded from the nuclear region. In all cases fluorescence arises from 1 mol % fluorescent lipid as described in Lewis et al. (1980).

that the NADPH oxidase is not activated uniformly either in individual macrophages or in phagosomes within the macrophages. However, we do not know whether the nonuniform distribution of nitro blue tetrazolium precipitate reflects a nonuniform NADPH oxidase stimulation among and within the macrophages or whether the nitro blue tetrazolium, which is membrane impermeable in its soluble form, is not made uniformly available to all of the phagosomes. Also, we do not know the degree of linearity of the nitro blue tetrazolium assay with respect to detection of O_2^-. The observable differences between DMPC- and DPPC-stimulated macrophages in the presence of nitro blue tetrazolium are in the same direction and of the same magnitude as the differences in respiratory burst as measured by oxygen consumption and 1-*C*-glucose oxidation. The latter methods measure the respiratory burst in a manner which is not subject to the potential ambiguities of the nitro blue tetrazolium assay but do not yield information about cell or phagosome heterogeneity.

A nitro blue tetrazolium precipitate was seen also when RAW264 macrophages phagocytized sheep red blood cells

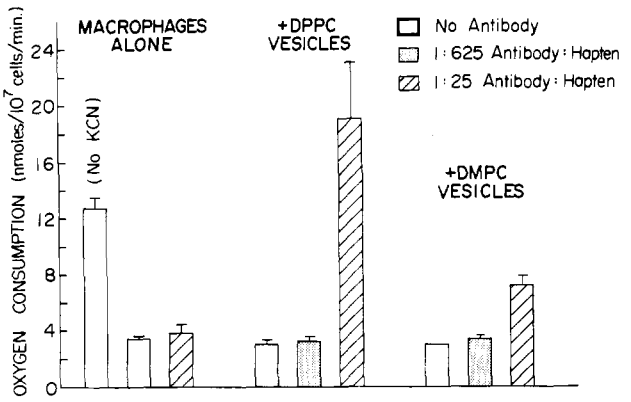

FIGURE 3: Oxygen consumption in the presence of 2.0 mM KCN by RAW264 macrophages stimulated with DMPC or DPPC vesicles with 1 mol % nitroxide spin-label phospholipid hapten. Antinitroxide antibody was supplied as a total IgG fraction from immunized rabbits. Total exposed hapten was 3.75×10^{-7} M. Bars show the mean ± range of duplicate determinations. For comparison, oxygen consumption in the presence of the soluble stimulator, phorbol myristate acetate, at 2 μg/mL was 36 ± 6 nmol $(10^7$ cells min$)^{-1}$.

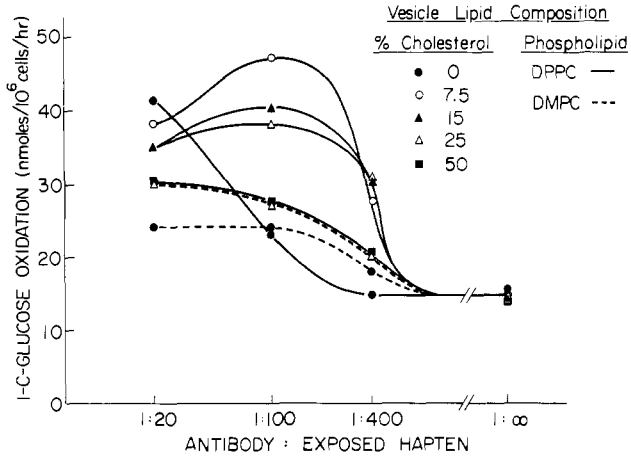

FIGURE 4: Effects of vesicle binary lipid composition on exogenous 1-*C*-glucose oxidation by RAW264 cells. The total amount of vesicle lipid was 0.020 μmol, and the "medium"-size fractions of the vesicles were used. Incubation was 1 h at 37 °C. Antibody was supplied as a total IgG fraction from immunized rabbits; exposed hapten was 1.2 $\times 10^{-7}$ M. Points show the mean of duplicate determinations at 1:20 and 1:400 and single observations at 1:100 and 0 antibody/exposed hapten ratios. The standard deviation was ~5% of measured values. Resting macrophages, in the absence of vesicles, oxidized 14.2 ± 0.4 nmol $(10^6$ cells h$)^{-1}$.

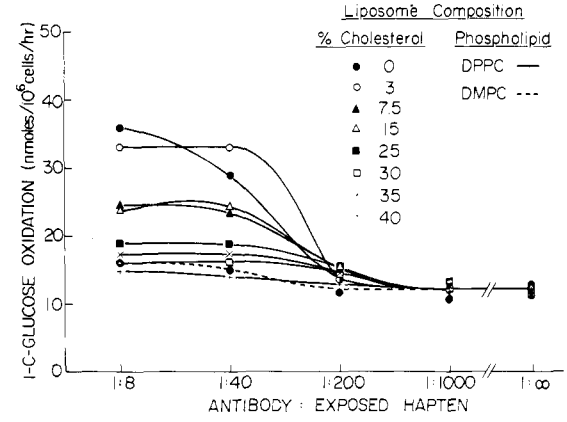

FIGURE 5: Effects of binary lipid composition of haptenated multilamellar liposomes on exogenous 1-*C*-glucose oxidation by RAW264 cells. The total amount of liposomal lipid was 0.1 μmol. Incubation was 1 h at 37 °C. Antibody was supplied as a total IgG fraction from immunized rabbits. Exposed hapten (~5% exposed) was 1.0×10^{-7} M. Points show the mean of duplicate determinations. The standard deviation of single observations was ~3% of the measured value. Resting cells without liposomes oxidized 11.1 ± 0.1 nmol $(10^6$ cells h$)^{-1}$ in the absence of and 11.8 ± 0.3 in the presence of the highest (1:8) antibody concentration.

coated with a density of rabbit anti-sheep-erythrocyte IgG antibody which gave maximum macrophage-dependent external release of ^{51}Cr. For this study specially constructed sealed glass chambers containing macrophages and targets were placed on a 37 °C stage for microscopic examination. Small distinct spots of nitro blue tetrazolium precipitate appeared near the membrane of the red blood cell which was the first to be phagocytized. A few mintues later this red blood cell was filled with diffuse blue precipitate. Over the course of 1 h, small dark spots of precipitate appeared on the membrane of a red blood cell which was phagocytized a few mintues after the first. Over the course of several hours, nitro blue tetrazolium precipitate appeared on the red blood cells in sequence. The nitro blue tetrazolium results, taken as a whole, suggest that the NADPH oxidase is compartmentalized and that these compartments may be stimulated independently.

The result that the respiratory burst, measured either by 1-*C*-glucose oxidation or by cyanide-insensitive oxygen consumption, is stimulated much more efficiently by antibodies bound to DPPC vesicles compared to DMPC suggested that the physical state of hapten and bound antibody might be a factor in the activation. To test further this hypothesis, we constructed membranes having various hapten mobilities by varying cholesterol in the vesicle membranes. Increasing cholesterol from 0 to 25 mol % in DPPC membranes has a marked effect on phospholipid diffusion at 38 °C (Copeland & McConnell, 1980; Owicki & McConnell, 1980; Rubenstein et al., 1979, 1980). Dipalmitoylphosphatidylcholine vesicles with 1 mol % nitroxide hapten and various amounts of cholesterol were prepared and used to measure activation of macrophage 1-*C*-glucose oxidation in the presence of specific antinitroxide antibody. Figure 4 shows that in the absence of antibody there is no respiratory activation by vesicles with or without cholesterol. At the low 1:400 antibody/exposed hapten ratio, activation is observed only with DMPC and DPPC vesicles containing cholesterol. As shown previously, the macrophages are unable to bind DPPC vesicles with this low antibody surface density at an appreciable rate (Lewis et al., 1980). Increasing the antibody/exposed hapten ratio to 1:100 results in optimal activation of cells by vesicles containing lipid other than pure DPPC. At this optimum, their order of effectiveness in activation of the respiratory burst decreases as the mole fraction of cholesterol is increased above 7.5 mol %. A further increase in the antibody/exposed hapten ratio results in a decrease in activation with the three membranes containing the lowest amounts of cholesterol. This effect occurs with DPPC vesicles without cholesterol at higher antibody/exposed hapten ratios (Figure 1). As observed previously, DPPC vesicles require high antibody for optimal stimulation, in accord with the high antibody/exposed hapten ratio necessary to obtain binding (Lewis et al., 1980). On the other hand, DMPC vesicles bind and stimulate with a lower antibody/exposed hapten ratio, but the high level of activation is never achieved.

To determine if these results can be explained by an artifact due to possible heterogeneous cholesterol composition in vesicles prepared by the ether injection method, we prepared multilamellar liposome mixtures with 1% nitroxide hapten and various amounts of membrane cholesterol. The liposomes and various amounts of antibody were incubated with macrophages and 1-*C*-glucose as in previous experiments. The results

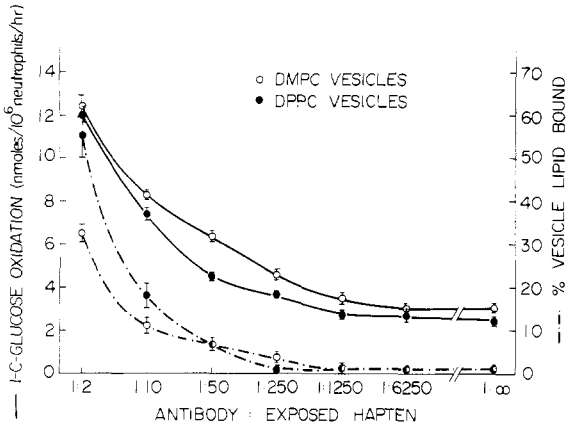

FIGURE 6: Neutrophil oxidation of exogenous 1-*C*-glucose and binding of DMPC or DPPC vesicles with 1 mol % nitroxide spin-label phospholipid hapten and various amounts of antinitroxide antibody. The total amount of vesicle lipid was 0.025 μmol, and the "large" vesicle size fractions were used. The incubation was 1 h at 37 °C. Antibody was supplied as affinity-purified rabbit antinitroxide IgG; exposed hapten was 1.5×10^{-7} M. Points show the range of duplicate determinations. Resting neutrophils oxidized 3.6 ± 0.2 nmol (10^6 cells h)$^{-1}$ and 3.9 ± 0.1 and 4.2 ± 0.1 without vesicles in the presence of 1:5 and 1:1 antibody, respectively.

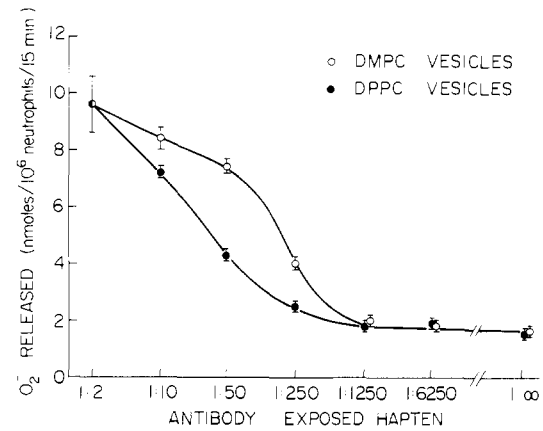

FIGURE 7: Superoxide release during a 15-min stimulation of neutrophils with DMPC or DPPC vesicles and antibody as in Figure 6. Controls without vesicles gave essentially the same resting rate and showed no increase at any of the antibody concentrations. Points show the range of duplicate determinations.

(Figure 5) are qualitatively similar to those in Figure 4 in that at the higher antibody/exposed hapten ratios, progressive increases of cholesterol concentrations result in a progressive decrease in activation of macrophages. For all cholesterol-containing liposomes, optimal activation occurs at the 1:40 antibody/exposed hapten ratio. Here, glucose oxidation is increased relative to the resting level by factors of 2.4 for DPPC liposomes containing 0% cholesterol, 2.7 for 3 mol % cholesterol, 2.0 for 7.5 mol % cholesterol, and 1.8 for 25 mol % cholesterol and still less for liposomes with higher levels of cholesterol or pure DMPC liposomes. Increasing the antibody/exposed hapten ratio to 1:8 causes a further increase only with DPPC membranes without cholesterol. The absence of completely parallel behavior of liposomes (Figure 5) and vesicles (Figure 4) with respect to macrophage activation may be related to uncontrolled variations of the shapes and sizes of the lipid membranes and possibly also to small uncontrolled variations in lipid composition.

We have also measured the response of human neutrophils to antibody-coated vesicles. Like macrophages, neutrophils bind both DMPC and DPPC vesicles in a specific antibody-dependent fashion, but neutrophils require 10–100-fold more antibody. The larger factor applies to DMPC vesicles since neutrophils showed no enhanced binding of antibody-coated DMPC vesicles. Figure 6 shows the antibody-dependent binding of haptenated DMPC and DPPC vesicles to neutrophils and the accompanying stimulation of 1-*C*-glucose oxidation. Glucose oxidation increases with higher antibody/exposed hapten ratios in a manner parallel to vesicle binding. Also in contrast to macrophages, we found no indication that vesicles with haptens of low lateral mobility caused more activation than vesicles having high lateral mobility. Instead, 1-*C*-glucose oxidation is slightly greater with DMPC vesicles compared to DPPC vesicles at all antibody concentrations. Measurement of O_2^- release from neutrophils confirmed these results. Resting neutrophils release 1.5 nmol of $O_2^-/10^6$ cells in 15 min (Figure 7). This rate increases progressively with either DPPC or DMPC vesicles when the antibody/exposed hapten ratio is elevated above the 1:250 level necessary to initiate binding. The decrease in O_2^- release at high antibody concentrations reported previously (Hafeman et al., 1979) was prevented in the present work by inclusion of 0.2 wt % bovine serum albumin in the aqueous medium. Without this protein, high concentrations of free antibody (or possibly small amounts of antibody aggregates) stimulate 1-*C*-glucose oxidation but not O_2^- release. This activation inhibits the subsequent surface activation and O_2^- release caused by antibodies bound to vesicles (D. G. Hafeman, J. W. Parce, and H. M. McConnell, unpublished observations).

The highest rate of O_2^- release is observed at the 1:2 antibody/exposed hapten ratio where both DMPC and DPPC vesicles stimulated neutrophils to release 9.6 nmol/10^6 cells in 15 min. At intermediate antibody levels, DMPC vesicles give rise to a higher response than DPPC vesicles. This effect may be related to a longer interval of time between cell activation and complete endocytosis of the DMPC vesicles. The enhanced O_2^- release caused by DMPC membranes is more pronounced if large multilamellar liposomes are used instead of unilamellar vesicles (Hafeman et al., 1979). However, when cytochalasin B is used to inhibit phagocytosis, the O_2^- release stimulated by antibody-coated DPPC liposomes is increased and the difference between DMPC and DPPC is abrogated (J. W. Parce, D. G. Hafeman, and H. M. McConnell, unpublished observations).

Discussion

In the accompanying paper we demonstrate that specific antibody-dependent binding of hapten-sensitized vesicles to RAW264 macrophages is more rapid for vesicles that are fluid at 37 °C (DMPC and cholesterol-containing DPPC) compared to solid DPPC vesicles, especially at low antibody/exposed hapten ratios. We believe this effect to be due to the higher rate of lateral diffusion of bound antibodies on these vesicles, as discussed in the companion paper (Lewis et al., 1980).

The present study examines the physical properties of these target membranes with respect to activation of these macrophages, subsequent to vesicle binding to the macrophages. We have demonstrated that the triggering of the macrophage respiratory burst by antibody-coated vesicles is a function of at least two additional variables. They are (1) antibody density on the vesicle surface and (2) the lipid composition of the vesicles. That macrophage activation requires a greater density of antibodies on the vesicle surface than is required for binding of the vesicles to these cells is most clearly demonstrated with DMPC vesicles. With these vesicles, maximal rates of vesicle binding are observed even with low antibody/exposed hapten ratios (Figure 1). The curve showing activation of the re-

spiratory burst displays a definite lag relative to the curve showing vesicle binding at various antibody/exposed hapten ratios. The respiratory burst, as monitored by 1-*C*-glucose oxidation, reaches a plateau with DMPC vesicles and a maxmimum with DPPC vesicles at the 1:50 antibody/exposed hapten ratio. This corresponds to an antibody surface density of $\sim 333/\mu m^2$ (Lewis et al., 1980). This number is similar to the density of Fc receptors on the macrophage membrane [assuming a macrophage surface area of $\sim 1000\ \mu m^2$ (Steinman et al., 1976)], as estimated from existing literature data on various macrophages and macrophage cell lines similar to RAW264. [A typical value obtained by using rabbit antibody is 400 000 receptors/macrophage (Segal & Hurwitz, 1977).]

Although the macrophages bind fluid vesicles with fewer antibodies, when sufficient antibody is added to obtain binding of the solid vesicles, activation is always much greater with solid DPPC compared to fluid DMPC vesicles. This is true over a wide range of antibody/exposed hapten ratios. When the effects of lipid composition and antibody density are taken together, the distinction between vesicle binding and activation of the respiratory burst is quite dramatic. For example, at the 1:1250 antibody/exposed hapten ratio where 50% of the DMPC vesicles bind and become phagocytized, the measured 1-*C*-glucose oxidation above the resting background is barely detectable. On the other hand, at a 25-fold higher specific antibody surface density on DPPC vesicles where 50% of the DPPC vesicles bind and are phagocytized, 1-*C*-glucose oxidation is some 5 times the resting level. The inescapable conclusion is that activation of the respiratory burst in the RAW264 macrophage is not simply a function of the quantity of vesicles bound and phagocytized. Recent physical–chemical studies of the properties of DPPC–cholesterol bilayer membranes using freeze–fracture electron microscopy, spin-label paramagnetic resonance, and pattern photobleaching recovery techniques have revealed that such membranes have a remarkable structure in a temperature range that includes 37 °C and at cholesterol concentrations between 0 and 20 mol %. There are alternating, approximately parallel domains of solid DPPC (with essentially constant widths of 150 Å) and fluid domains containing 20% cholesterol whose widths increase with increasing average cholesterol concentration (Copeland & McConnell, 1980; Owicki & McConnell, 1980; Rubenstein et al., 1979, 1980). For example, at 10 mol % cholesterol, the membrane surface is approximately 50% fluid and 50% solid in alternating bands of widths ~ 150 Å. This confers unusual properties on these membranes; long-range diffusion (i.e., on the order of 1 μm) is very slow for any path that crosses the solid bands, except above 20 mol % cholesterol where the solid bands disappear. Thus, there is an order of magnitude jump in long-range lateral diffusion in typical samples at 20 mol % cholesterol. On the other hand, short-range diffusion in the fluid bands remains rapid. This property of such lipid bilayers is manifested in the present study as membranes which are especially effective in activating the respiratory burst. Evidently antibodies bound to haptens in the fluid domains give high vesicle binding rates, and antibodies bound to haptens in the solid domains yield enhanced respiratory activation. Therefore, lipid membranes composed of DPPC and <20 mol % cholesterol give particularly high activation rates relative to membranes containing no solid or no fluid lipids, except for solid membranes at high antibody surface densities where the binding rate is not limiting.

The present work raises the interesting problem as to why, per vesicle bound, lipid membranes containing some solid DPPC are particularly effective in antibody-dependent respiratory activation. There may be a general, qualitative solution to this problem. When one compares lipid haptens in solid membranes with lipid haptens in fluid membranes, the lipid haptens in the fluid membranes are clearly more like free haptens in three-dimensional isotropic solution than are the haptens in solid membranes. (In the same manner phospholipid haptenic groups separated from the glycerol backbone by a large spacer must be more like free haptens in solution than are haptenic groups separated from the glycerol backbone by a short spacer.) In general, antibodies bound to free haptens in solution do not activate antibody-dependent effector components of the immune system. Antibodies bound to haptens that are cross-linked to one another do activate these components, possibly due to conformational changes in antibody structure. If this view is correct, it implies that a larger fraction of antibodies bound to solid DPPC undergoes a conformational distortion than is the case for antibodies bound to fluid membranes. [For a discussion of antibody conformation changes and their possible relevance to the triggering of antibody-dependent immune responses, see reviews by Metzger (1974, 1979).] In considering the plausibility of such conformation changes, one must bear in mind that the haptenic groups are very small compared to the size of an IgG antibody molecule and that it is extremely unlikely that the two hapten combining sites in an undistorted IgG molecule could match perfectly with two haptens confined to a plane. Thus, the combination of *two* haptens with two antibody combining sites [which is known to be the case for the present system (Parce et al., 1979)] requires distortion of antibody, membrane, or both. The accommodation of membrane to a given antibody conformation is clearly more facile in the case of a fluid membrane than a solid membrane. Thus, a larger fraction of distorted antibodies are expected on the surface of solid membranes under equilibrium conditions. This implies that antibodies bind to solid DPPC membranes with an affinity that is less than the binding to fluid DMPC membranes. The studies of this binding that have been carried out show that the binding affinities to the two membranes are of the same order of magnitude but that the observed differences are in the direction indicated above (Humphries & McConnell, 1980).

In the present and accompanying paper we have shown that there is a clear distinction between the kinetics of RAW264 vesicle binding and the kinetics of respiratory activation, irrespective of detailed molecular interpretations. In the case of neutrophils, no major difference is observed in the 1-h binding to DMPC and DPPC vesicles, and the degree of respiratory activation is found to parallel closely the vesicle binding (cf. Figure 6). Since for neutrophils under these conditions, a high antibody density on the vesicle surfaces is required to achieve both binding and respiratory activation, it has not been possible to study these processes separately. Additional experiments under other conditions might show that neutrophils can distinguish between fluid and solid vesicles, with respect to both binding and respiratory activation. In view of our present studies of macrophage binding and activation and earlier studies of the binding and activation of the first component of complement, we anticipate that an independence of effector binding and activation on target membrane "fluidity" will be the exception rather than the rule.

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