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Immunology

Specific antibody-dependent interactions between macrophages and lipid haptens in planar lipid monolayers

(Fc receptors/respiratory burst/planar membrane/fluorescence)

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ABSTRACT **The binding of guinea pig peritoneal macrophages to planar lipid monolayers on alkylated glass is shown to be highly specific, requiring lipid hapten in the monolayer and specific anti-hapten IgG antibody. This is true for both "fluid" and "solid" monolayer membranes, in which the lateral diffusion coefficients of fluorescent lipid probes and bound fluorescent antibodies differ by at least two orders of magnitude. The region of the (macrophage membrane)–(monolayer membrane) contact is readily observed by using an epifluorescence microscope and fluoresceinated IgG antibodies or fluorescent lipids. The fluorescence intensity of IgG antibodies bound to lipid haptens in fluid monolayer membranes in the region of the (macrophage membrane)–(monolayer membrane) contact was significantly enhanced in the early stages of binding (first 10 min at 24°C), due to a diffusive flux of the fluorescent antibodies into the region of membrane–membrane contact. Cellular activation takes place immediately during a 10-min warm-up to 37°C and can be recognized by rapid symmetrical cell spreading, the formation of "black holes" around the cells (probably due to superoxide-facilitated photochemical bleaching of the fluorophore), and the release of the lysozomal enzyme cathepsin B. Specific antibody-dependent [1-^{14}C]-glucose oxidation by these macrophages on fluid and solid monolayers is quite similar to that reported previously for fluid and solid bilayer vesicle target membranes. These results are significant for understanding the molecular interactions between membranes that are necessary for a macrophage cytotoxic response.**

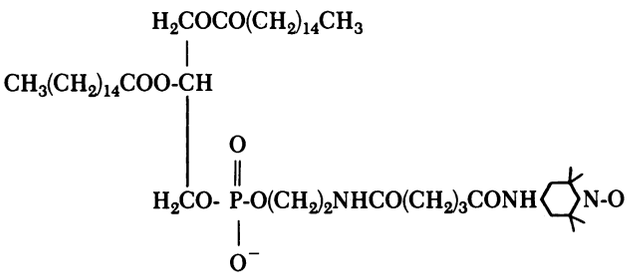
Reconstituted lipid bilayer membranes containing specific antigens or lipid haptens have been used successfully in a number of studies of cell surface recognition by cellular and humoral components of the immune system (for examples of recent work and references to earlier literature, see ref. 1). Such reconstituted lipid bilayer membranes are usually in the form of multilamellar liposomes or single-shell vesicles. In some studies the question has arisen as to whether the spherical shape (e.g., radius of curvature $\approx 0.5\ \mu m$), membrane flexibility, or both, of these vesicles or liposomes might play a significant role when cell membrane-reconstituted target membrane contact is involved in binding and triggering (2–4). For this reason we have been motivated to study the specific binding and triggering of cellular components of the immune system by lipid monolayer membranes attached to a planar glass surface. In the present work, the specific antibody-dependent interactions of macrophages with the supported membranes have been studied, and the results have been compared with previous studies of similar interactions of macrophages with vesicle membranes (2–4).

A second motivation for the present work has been a desire to use the fluorescence microscope to visualize molecular events that take place at the interface between a cellular membrane and a reconstituted membrane during the course of a specific functional interaction between the two membranes. A monolayer target membrane attached to a planar glass surface provides an ideal configuration with which to make such observations.

MATERIALS AND METHODS

Macrophages. Resident peritoneal cells were collected from Hartley guinea pigs (EZH Caviary, Williams, CA). The ether-anesthetized animals were sacrificed by cardiac puncture followed by cervical dislocation. The cells were collected in 200 ml of cold washing buffer (6.8 g of NaCl, 0.4 g of KCl, 0.14 g of NaH_2PO_4, 1.0 g of D-glucose, 6.0 g of Hepes per liter; pH 7.4) with 1 mM EDTA. The cells were concentrated by sedimentation and the macrophages were purified by step-density gradient centrifugation on Ficoll/Hypaque separation medium (ρ = 1.077 g/ml) as described by Böyum (5). The cell preparation ($\approx 4 \times 10^7$ per animal) contained ≈90% macrophages as indicated by staining with crystal violet. Cell viability, as determined by trypan blue exclusion, was >95%. Macrophages 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_2HPO_4, 5.6 mM glucose, 120 mM NaCl, and 0.2% bovine serum albumin (Calbiochem, fatty-acid poor); pH 7.4] and were kept on ice prior to use.

Hapten and Lipids. The dipalmitoyl phospholipid nitroxide spin-label hapten I was synthesized by D. Carter according to a modification of Brûlet's synthesis (6) developed by L. Smith and D. Torney in this laboratory (unpublished experiments).



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The fluorescent phospholipid probe *N*-(4-nitrobenzo-2-oxa-1,3-diazolyl)phosphatidylethanolamine (NBD-PtdEtn), prepared from dimyristoyl phosphatidylethanolamine, was purchased from Avanti Biochemicals. Dipalmitoyl phosphatidylcholine (Pam_2-PtdCho) and dimyristoyl phosphatidylcholine (Myr_2-PtdCho), both grade A, were purchased from Calbiochem and stored at 10 mM in methanol at −20°C.

Abbreviations: Myr_2-PtdCho, dimyristoyl phosphatidylcholine; Pam_2-PtdCho, dipalmitoyl phosphatidylcholine; NBD-PtdEtn, *N*-(4-nitrobenzo-2-oxa-1,3-diazolyl)phosphatidylethanolamine.

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Unilamellar phospholipid vesicles were prepared by the ether injection method described previously (2). Vesicles (≈1 μm diameter) were resuspended in 0.15 M NaCl at 0.25 mM lipid after centrifugation at 12,000 × *g* for 30 min. All phospholipid vesicles contained 1 mol % nitroxide spin-label phospholipid hapten, 1 mol % NBD-PtdEtn, and 0.25 mol % [1-^{14}C]Pam$_2$-PtdCho (Amersham).

Antibodies. Anti-nitroxide IgG was prepared by immunizing rabbits according to a procedure described by Humphries and McConnell (7), with the modification that an alum precipitate of 100 μg of spin-labeled keyhole limpet hemocyanin was used in the boosting intravenous injections.

Lipid Monolayers. Clean microscope cover glasses were treated with octadecyltrichlorosilane (Petrarch Systems, Levittown, PA), washed with water, and passed through a lipid monolayer at the air–water interface so as to produce a lipid monolayer on the treated surface. The slide thus treated remained under an aqueous phase in subsequent manipulations so that the polar head groups were always exposed to the aqueous phase. The pressure–area–temperature conditions for forming fluid and solid monolayers along with a description of other physical properties of these monolayers will be described in detail elsewhere. Diffusion coefficients of fluorescent molecules bound to this supported monolayer were determined by a periodic pattern photobleaching technique (8). The pattern photobleaching as well as all other optical microscopic measurements were carried out by using a Zeiss Photomicroscope III, equipped with a 37°C stage and otherwise modified so as to accomodate a cooled photomultiplier and an image intensifier (Nitek). All monolayers contained 1 mol % hapten I unless otherwise noted.

[1-^{14}C]Glucose Oxidation. The stimulation of macrophage [1-^{14}C]glucose oxidation by haptenated lipid vesicles and anti-hapten antibodies was measured as described (3). Glucose oxidation by macrophages bound to the haptenated phospholipid monolayer was similarly determined except that the lipid monolayer on an 18-mm circular cover glass was placed in a 10-ml beaker (never reexposing the monolayer to any gas phase). The water was replaced by a final volume of 1.1 ml of 0.15 M NaCl by successive dilution. Anti-nitroxide antibody and 0.71 μCi of [1-^{14}C]glucose (Amersham, 50–60 μCi/μmol; 1 Ci = 3.7×10^{10} becquerels) in a total volume of 1.1 ml of cell buffer were added with stirring and incubated for 30 min. Macrophages (5×10^5) were added and incubated at 37°C for 1 hr without stirring. Respired CO_2 was collected as previously in experiments with phospholipid vesicles, using an appropriately sized rubber stopper for the chamber top (3).

Incubations for Microscopy and Staining for Cathepsin B. The lipid monolayer on an 18-mm square no. 2 cover glass was mounted in a 60 × 15 mm glass Petri dish with two double-thickness strips of 1/4-inch double-coated tape (3M, St. Paul, MN). The mounting was carried out under water so as to avoid the disruptive effect of an air–water interface on the monolayer.

For cell studies, water was replaced by cell buffer. Sixty microliters of fluoresceinated anti-nitroxide IgG was added and incubated for 10 min. The monolayer was washed with cell buffer and 100 μl of the macrophage suspension was added. The Petri dish was inverted and the cells were allowed to sediment onto the monolayer for 10 min. The dish was then righted and excess cell buffer was added for observation with a water immersion objective. Fig. 1 shows the mounted preparation as employed for phase contrast and epifluorescence microscopy. Temperature (37°C) was monitored with a small thermoresistor placed in the surrounding aqueous medium.

Adherent cells were stained for cathepsin B by washing the monolayer with one drop of 0.15 M NaCl and flushing with 100 μl of 1 mM benzyloxycarbonyl-alanyl-arginyl-arginyl-4-methoxy-2-napthylamine/1 mM 5-nitrosalicylaldehyde/1 mM dithiothreitol/0.2 mM EDTA in 0.2 M ammonium acetate, pH 6.0, as described by Dolbeare and Vanderlaan (9). After 10-min incubation at 37°C the stain was replaced by 0.15 M NaCl for microscopic observation.

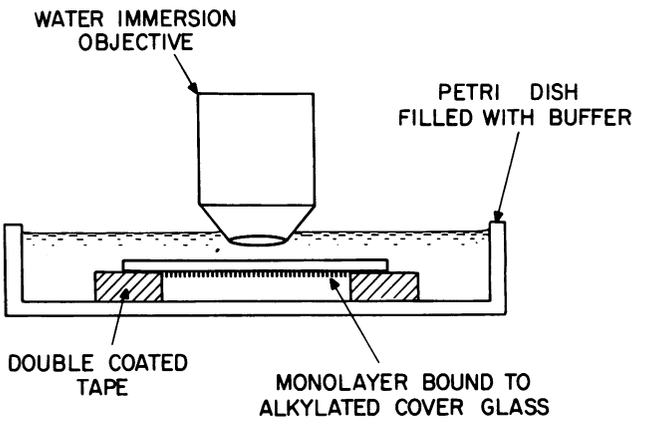

FIG. 1. Monolayer membrane as mounted to facilitate fluorescence photobleaching and microscopic observation of adherent cells. Alternatively, "monolayer-up" preparations can be used with a more cumbersome procedure for exchanging the aqueous phase.

RESULTS

Fluoresceinated rabbit anti-nitroxide IgG bound to the lipid hapten in the monolayer showed a uniform fluorescence over the entire slide. In a number of experiments in which the buffer solution surrounding the antibodies was changed a number of times, there was no significant loss in fluorescence intensity of bound antibodies. In contrast, antibody fluorescence on the monolayer was not observed when lipid hapten was omitted from the monolayer membrane. The number of antibodies bound to the fluid and solid membranes appear to be comparable as judged by fluorescence intensity. Under the conditions of our experiments the Myr$_2$-PtdCho membranes were judged to be relatively "fluid" because the lateral diffusion coefficients of a fluorescent lipid probe (NBD-PtdEtn) and fluoresceinated antibodies were always at least two orders of magnitude greater than the diffusion coefficient of these fluorescent molecules in the Pam$_2$-PtdCho membranes. Myr$_2$-PtdCho and Pam$_2$-PtdCho membranes were removed from the air–water interface at surface pressures of 35 and 39 dynes/cm, respectively, and at 25.7°C. The diffusion coefficient of the fluoresceinated IgG in the fluid Myr$_2$-PtdCho membranes was 3.5×10^{-9} cm^2/sec at 37°C. For the solid Pam$_2$-PtdCho membranes, the diffusion coefficient of fluoresceinated IgG was $<10^{-11}$ cm^2/sec. Our measured diffusion coefficients reflect the differences reported previously for solid and fluid membranes, except that the absolute values of the diffusion coefficients measured here are roughly an order of magnitude smaller than those reported previously for antibodies bound to haptenated liposomes (10). We have found that this difference is due to the binding of serum albumin to the monolayer surface, an effect that will be described in more detail elsewhere.

The binding of the guinea pig peritoneal macrophages to the monolayer was strictly antibody dependent at 24°C. In the absence of antibodies the number of monolayer-bound macrophages was $1/10^3$ to $1/10^4$ times as much as in the presence of antibodies. Bound macrophages began spreading rapidly during the warm-up from 24°C to 37°C. Unlike macrophages nonspecifically stuck to bare glass slides, macrophages specifically

bound to hapten-bound antibodies spread nearly uniformly in all directions, on both fluid and solid monolayers (Fig. 2).

The specific antibody-dependent binding of macrophages to lipid–hapten monolayer membranes was accompanied by striking changes in fluorescence intensity in the region of (monolayer membrane)–(macrophage membrane) contact. Immediately upon microscopic observation (10 min after incubation at 24°C) we noted a significant enhancement of uniform fluorescence intensity originating in the plane of the monolayer, in the region of contact between macrophage and the lipid monolayer. This enhancement of fluorescence intensity is much more pronounced for the fluid monolayers and is therefore attributed to the diffusion of monolayer-bound antibodies to the region of the monolayer–macrophage membrane contact, where Fc domain–Fc receptor bonds are formed so as to trap the fluorescent antibodies in this region. This trapping rate must be more rapid for the fluid monolayers. The enhancement of fluorescence intensity is quite striking, precedes macrophage spreading, and remains under those macrophages that do not spread (Fig. 3). The macrophage spreading process appears to be more rapid on the solid membranes than on the fluid membranes.

The specific antibody-dependent spreading of the macrophages on lipid–hapten-containing fluid and solid monolayers was accompanied by the formation of "black holes." That is, within 2 or 3 sec of illumination with low-intensity excitation light, the fluorescence intensity in the region of the spreading cells was bleached compared to the fluorescence intensity of the surrounding monolayer. This enhanced photobleaching is thought to be due to reactive oxygen species (possibly O_2^-) released by triggered macrophages. The black holes were more numerous and better defined on the solid monolayer, probably because of the greater lateral diffusion of bleached and nonbleached fluorescently labeled antibodies on fluid monolayers. During this process the lipid monolayer appears unaffected. When monolayers containing the fluorescent lipid probe NBD-PtdEtn were used together with nonfluorescent antibodies, the macrophages showed the same morphological and biochemical parameters of activation, but the monolayer remained uniformly fluorescent.

After 30-min to 1-hr incubation at 37°C we have also observed "black tracks." Such "black tracks" are permanent records of the translational motion of the macrophages on the lipid monolayer with bound fluorescein-labeled antibodies that have been continuously illuminated with very low intensity excitation light during the 37°C incubation. Directed motion of individual cells was confirmed by phase-contrast microscopy and time-lapse photomicroscopy. The antibody-dependent, cell translation appeared to be in random directions for individual cells (Fig. 4). This cell morphology and translation were observed on both fluid and solid monolayers but were not observed in the absence of anti-nitroxide antibodies (see Fig. 2 *Lower*).

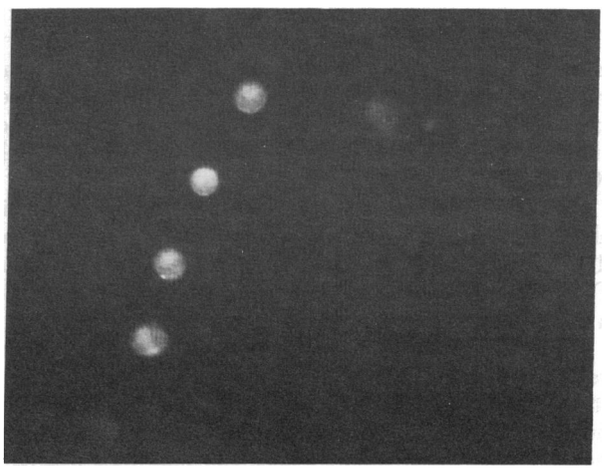

FIG. 3. Epifluorescence photomicrograph showing increased intensity of monolayer-bound fluorescent IgG in the region of cell contact after 37°C incubation for 1 hr on a Myr_2-PtdCho monolayer. Spread macrophages in the same fluid are not observed due to rapid photobleaching of fluorescein in the vicinity of these cells (see *Results*). (×640.)

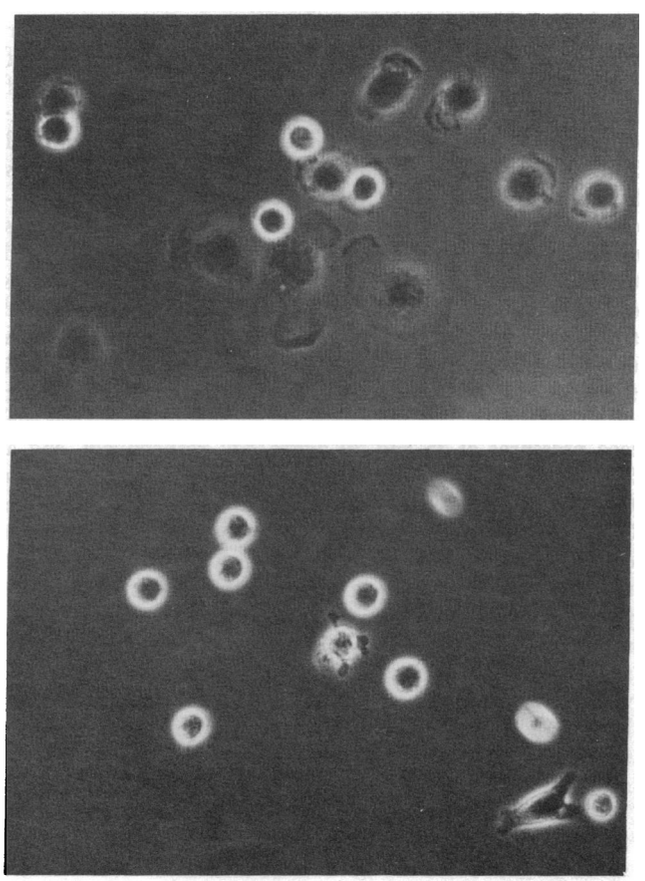

FIG. 2. Phase-contrast photomicrographs of macrophages on planar Pam_2-PtdCho lipid monolayers after 30-min incubation at 37°C in the "monolayer up" configuration. (*Upper*) With a 1:500 dilution of anti-nitroxide IgG; (*Lower*) without antibody. (×640.)

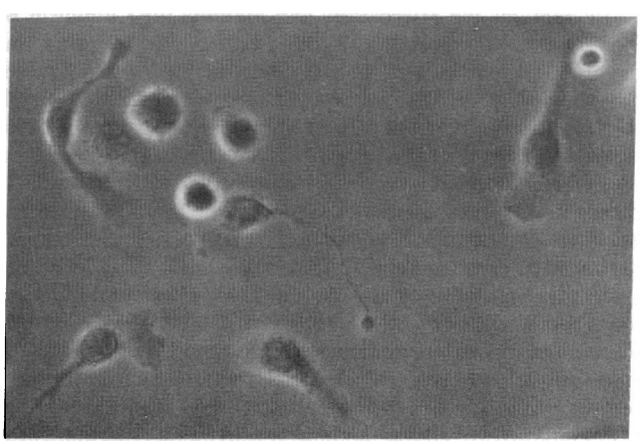

FIG. 4. Phase-contrast photomicrograph showing macrophages undergoing translation on a Pam_2-PtdCho monolayer with specifically bound IgG (1:500 dilution) after incubation for 90 min at 37°C. (×640.)

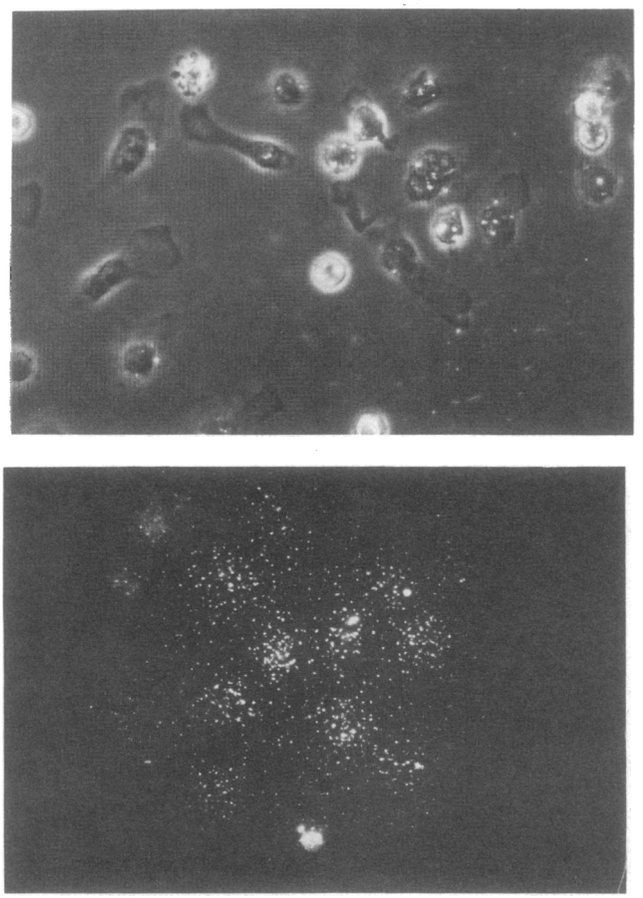

FIG. 5. Cathepsin B staining of macrophages adherent to a Pam_2-PtdCho monolayer with bound antibodies after 15-min incubation at 37°C. The yellow-orange granules of fluorescent precipitate formed by the enzyme are localized both inside the cells and outside the cells (associated with the monolayer). (*Upper*) Combined phase-contrast and epifluorescence photomicrograph taken in the median focal plane of the cells. [Note the decrease in the number of fluorescent (light) granules in spread cells compared to nonspread cells.] (*Lower*) Epifluorescence photomicrograph taken in a focal plane between the monolayer-coated cover glass and adherent macrophages, showing accumulation of fluorescent precipitate in this region. (×640).

Spreading of macrophages on the monolayer was also associated with the extracellular release of the lysozomal enzyme cathepsin B. The cathepsin B activity was visualized with fluorescence microscopy by using the method of Dolbeare and Vanderlaan (9). The action of cathepsin B on an appropriately designed substrate yields a fluorescent precipitate (see *Materials and Methods*). In the case of macrophages spread on the monolayer, this precipitate is localized partially in the monolayer plane and partially within the cell (Fig. 5). When the macrophages are stuck to a bare glass surface the fluorescent precipitate is completely localized within the cell.

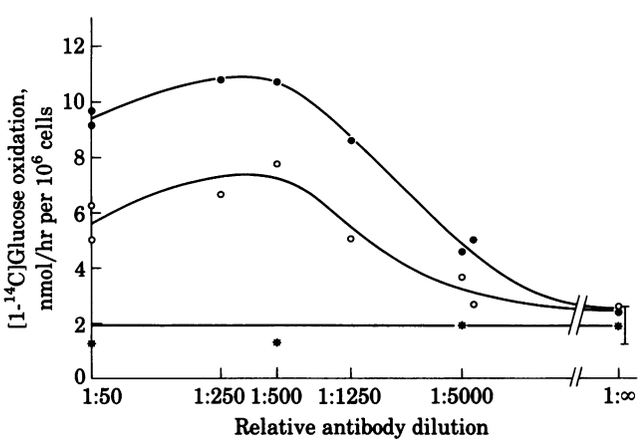

FIG. 6. Oxidation of exogenous [1-^{14}C]glucose by guinea pig macrophages on hapten-bearing Myr_2-PtdCho (○) or Pam_2-PtdCho (●) monolayers as a function of added anti-nitroxide IgG. *, Control with antibody alone.

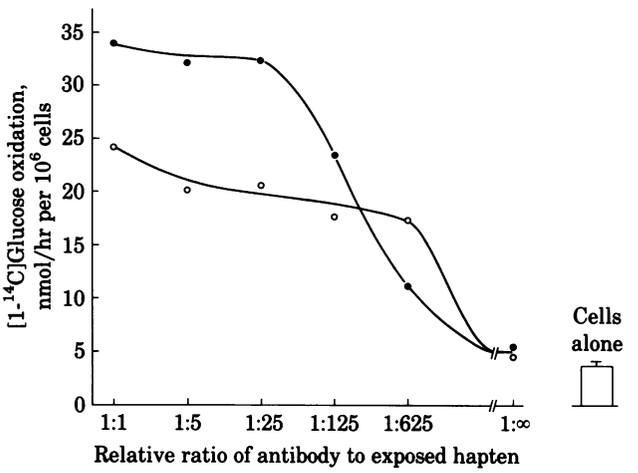

FIG. 7. Oxidation of exogenous [1-^{14}C]glucose by guinea pig macrophages during incubation with Myr_2-PtdCho (○) or Pam_2-PtdCho (●) vesicles with 1 mol % hapten I and various densities of bound anti-nitroxide IgG.

The rate of oxidation of [1-^{14}C]glucose by 5×10^5 macrophages that were allowed to settle onto lipid monolayers was measured. The results are given in Fig. 6, and they demonstrate an enhanced rate of glucose oxidation that requires both the haptenated monolayer and specific anti-nitroxide antibody. There is a clear maximum in glucose oxidation at the same concentration of specific anti-nitroxide IgG for both solid and fluid membranes in the presence of antibodies. Glucose oxidation was stimulated more effectively by the solid membranes relative to the fluid. At the optimal antibody concentration, glucose oxidation was increased 3.4-fold by Myr_2-PtdCho and 5.5-fold by Pam_2-PtdCho monolayers relative to resting cells (Fig. 6).

[1-^{14}C]Glucose oxidation by these macrophages was also measured by using lipid vesicles containing the same lipids and lipid hapten, in the presence of specific antibodies. The results are given in Fig. 7. The results are quite similar to those reported previously for a mouse macrophage cell line (3).

DISCUSSION

The present work was motivated by a desire to use a chemically and physically well-defined planar model membrane surface that serves as a target for specific cell surface recognition. As described in *Results*, we have achieved this goal both for specific antibody and hapten-dependent binding and for activation of guinea pig macrophages by lipid monolayers. The cellular response of the guinea pig macrophages to monolayers is both quantitatively and qualitatively remarkably similar to the responses of macrophages to lipid bilayer vesicles containing lipid haptens. This is demonstrated, first, by the requirement of lipid hapten and specific antibody for macrophage binding and activation of the respiratory burst and, second, by the dependence of this activation on lipid fluidity (2, 3). One subtle difference is notable: in the previous experiments with vesicles (and also in the vesicle experiments reported here in Fig. 7) the fluid lipid membranes are more effective in stimulating the respiratory

burst at low bound antibody density than are the solid membranes. This effect at low antibody densities has been shown to be due to the faster binding kinetics for the fluid vesicle targets (2, 3). In the present work with monolayers the effects of binding kinetics are essentially eliminated, because the macrophages settle onto the solid and fluid monolayers at the same rate.

Another aspect of the present work concerns the minimal requirements for specific antibody-dependent cellular triggering. In previous work it has been emphasized that antibodies bound to lipid haptens in lipid bilayer membranes can trigger a number of cellular responses. These antibody molecules appear to be bound in a random, mobile, non-clustered fashion by all physical measurements carried out to date, including lateral diffusion studies (10), freeze-etch electron microscopy (11), and electron paramagnetic resonance of the spin-label hapten (6). These results constitute *prima facie* evidence that the presence of aggregated Fc antibody domains is *not* a necessary prerequisite for antibody-dependent cell triggering. Experimental evidence suggesting that such antibody clustering (to induce receptor clustering) *is* necessary has been reviewed by Metzger (12, 13). A remaining issue has been the question as to whether the spherical geometry of the vesicle target could effectively crosslink antibodies in the target and receptors in the effector cell by a "focusing" interaction. The present work eliminates this issue.

Still another aspect of the present study is the demonstration that a functional contact between two membranes, one reconstituted and the other a natural membrane, can be visualized and studied with high-resolution optical fluorescence microscopy. This opens the way to the study of molecular motions of functional molecules shared by two membranes.

Finally, the highly specific antibody interaction between the macrophage membrane and the monolayer membrane suggests that the inclusion of other specific cell recognition molecules in monolayer membranes can be used to study additional immunological as well as tissue- and organ-specific membrane–membrane interactions.

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