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# Interaction of Antibodies with Fc Receptors in Substrate-Supported Planar Membranes Measured by Total Internal Reflection Fluorescence Microscopy†

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ABSTRACT: A procedure for constructing substrate-supported planar membranes using membrane fragments isolated from the macrophage-related cell line J774A.1 is described. Total internal reflection (TIR) fluorescence microscopy is employed to demonstrate that fluorescently labeled Fab fragments of a monoclonal antibody (2.4G2) with specificity for a murine macrophage cell-surface receptor for IgG (moFc$_\gamma$RII) bind to the planar model membranes. These measurements show that the planar membranes contain moFc$_\gamma$RII and yield a value for the association constant of 2.4G2 Fab fragments with moFc$_\gamma$RII equal to $(9.6 \pm 0.4) \times 10^8$ M$^{-1}$ and indicate that the surface density of reconstituted moFc$_\gamma$RII is $\sim$50 molecules/$\mu$m$^2$. In addition, TIR fluorescence microscopy is used to investigate the Fc-mediated competition of unlabeled, polyclonal murine IgG with labeled 2.4G2 Fab fragments for moFc$_\gamma$RII in the planar membranes. These measurements indicate that the reconstituted moFc$_\gamma$RII recognized by 2.4G2 Fab fragments also retains the ability to bind murine IgG Fc regions and yield a value for the association constant of polyclonal murine IgG with moFc$_\gamma$RII equal to $(1-5) \times 10^5$ M$^{-1}$. This work represents one of the first applications of TIR fluorescence microscopy to specific ligand-receptor interactions.

One mechanism of immunological defense is phagocytosis, in which macrophage cells ingest and destroy pathogenic organisms (Riches et al., 1988). In receptor-mediated phagocytosis, Fc receptors on a macrophage cell surface recognize IgG-coated targets and signal the macrophage to ingest the target (Unkeless et al., 1988). Murine macrophages have at least three distinct cell-surface IgG Fc receptors, and at least one of these receptors (moFc$_\gamma$RII) has been implicated in the phagocytotic process (Unkeless et al., 1981, 1988). Biochemical and immunological characterization of moFc$_\gamma$RII has been facilitated by the development of a rat-mouse hy-

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bridoma that secretes monoclonal antibodies specific for the receptor (2.4G2) (Unkeless, 1979) and by subsequent receptor purification (Mellman & Unkeless, 1980) and DNA sequencing (Hibbs et al., 1986; Ravetch et al., 1986; Lewis et al., 1986). However, a quantitative understanding of the role of moFc$_\gamma$RII–IgG interaction and transport in the macrophage–target contact region during the onset of receptor-mediated phagocytosis will require application of physicochemical techniques.

Information about the interaction of soluble ligands with membrane receptors can be obtained by examining model cell membranes deposited on transparent planar substrates with techniques in fluorescence microscopy. Substrate-supported planar membranes have previously been used in the investigation of a range of membrane processes, including T cell, macrophage, and basophil response to external stimuli (McConnell et al., 1986; Thompson & Palmer, 1988; Thompson et al., 1988). A technique in fluorescence microscopy that is particularly well suited to supported planar membranes is total internal reflection (TIR)[1] fluorescence microscopy (Axelrod et al., 1984). In this technique, a laser beam that is totally internally reflected at the planar membrane/solution interface creates a thin layer of illumination in the solution adjacent to the membrane called the evanescent field. The evanescent field selectively excites surface-bound fluorescent molecules and has been used in fluorescence microscopy to examine cell–substrate contact regions (Lanni et al., 1985; Nakache et al., 1986) and to measure surface binding kinetics (Burghardt & Axelrod, 1981; Thompson & Axelrod, 1983), orientation distributions (Thompson et al., 1984a), and protein–protein interactions (Watts et al., 1986). Among the proposed applications of TIR fluorescence microscopy is the measurement of equilibrium binding constants for weakly bound ligands specifically interacting with receptors on a surface (Axelrod et al., 1984).

Described in this work is the development of a procedure for constructing substrate-supported planar membranes that contain moFc$_\gamma$RII. TIR fluorescence microscopy is employed to investigate the binding of monoclonal anti-moFc$_\gamma$RII Fab fragments and also polyclonal murine IgG with moFc$_\gamma$RII in the planar membranes. The described work represents one of the first applications of TIR fluorescence microscopy to the measurement of specific ligand–receptor interactions.

## Materials and Methods

*Cells.* 2.4G2 hybridoma cells, which secrete antibodies specific for moFc$_\gamma$RII, were obtained as a generous gift from J. C. Unkeless of Mt. Sinai Medical School and from B. Diamond of the Albert Einstein College of Medicine. MAR18.5 hybridoma cells, which secrete murine IgG2a antibodies specific for rat immunoglobulin $\kappa$ light chains, were obtained from the American Type Culture Collection (Rockville, MD). The macrophage-related cell line J774A.1 was obtained from the University of North Carolina Tissue Culture Facility. Cells were maintained in culture in DMEM/F12 medium supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU penicillin G, 100 $\mu$g/mL streptomycin, and 5% (MAR18.5, J774A.1) or 7–10% (2.4G2) heat-inactivated (30 min, 56 °C) fetal calf serum (Flow Laboratories, McLean, VA).

*Antibody Purification, Fragmentation, and Fluorescent Labeling.* Intact rat and murine IgG (Sigma Chemical Co., St. Louis, MO), murine IgG (Fab)'$_2$, rat IgG Fab, alkaline phosphatase conjugated goat anti-(murine IgG) IgG (Fab')$_2$ (Jackson ImmunoResearch, Inc., West Grove, PA), and tetramethylrhodamine-labeled rabbit anti-(rat IgG) IgG (Fab')$_2$ (Southern Biotechnology Associates, Birmingham, AL) were obtained commercially. MAR18.5 antibodies were purified from cell culture supernatants by protein A affinity chromatography, and 2.4G2 antibodies were purified from cell supernatants either by goat anti-(rat IgG) affinity chromatography, by MAR18.5 affinity chromatography, or by both, as noted. Protein-coupled Sepharose 4B was obtained commercially (protein A and anti-rat IgG, Sigma) or prepared according to standard methods (MAR18.5; Mishell & Shiigi, 1980). Washing and elution buffers[2] were as follows: anti-(rat IgG), TBS, glycine buffer; MAR18.5, TBS, citrate buffer; protein A, bicarbonate buffer, citrate buffer.

2.4G2 fragments were produced by treating intact 2.4G2 IgG (0.1–0.5 mg/mL) with preactivated (15 min, 37 °C) papain (Worthington Biochemicals, Freehold, NJ) at a 1:100 w/w ratio of papain to 2.4G2 for 3 h at 37 °C in digestion buffer. Papain was removed by adsorbing the resulting mixture on a DEAE-52 column equilibrated in Tris buffer and then eluting with TBS. 2.4G2 Fab fragments were purified from Fc fragments with MAR18.5 affinity chromatography using the same conditions as those used for intact 2.4G2 IgG purification.

Intact and Fab fragments of 2.4G2 antibodies and rat IgG were labeled with tetramethylrhodamine isothiocyanate (Molecular Probes, Inc., Junction City, OR) as described (Mishell & Shiigi, 1980). The concentration of labeled antibodies (denoted by R-) and the molar ratios of tetramethylrhodamine to antibody (0.3 to 2.5) were determined spectrophotometrically. All antibodies and antibody fragments, both fluorescently labeled and unlabeled, were chromatographed on Sephadex G25 in PBS and passed through a 0.22-$\mu$m filter before use. For use with planar membranes, labeled 2.4G2 and rat IgG Fab fragments were further purified from unreacted dye by MAR18.5 affinity chromatography, and unlabeled antibodies and antibody fragments were centrifuged at 100000$g$ for 2 h. The relative fluorescence of different preparations of labeled antibodies was estimated on a spectrofluorometer (8000C, SLM, Urbana, IL) with excitation and emission wavelengths equal to 550 and 580 nm, respectively. Characterization of antibodies by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[1] was performed according to standard methods (Mishell & Shiigi, 1980).

*Assay for Rat $\kappa$ Light Chains.* Enzyme immunoassay for rat immunoglobulin $\kappa$ light chains was performed by using a BioDot microfiltration apparatus according to the manufacturer's procedure (Bio-Rad, Richmond, CA). Nitrocellulose was sequentially treated with bovine serum albumin (BSA, Sigma), 2.4G2 antibodies, or polyclonal rat IgG; MAR18.5 antibodies or polyclonal murine IgG; alkaline phosphatase

[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TIR, total internal reflection.

[2] The compositions of buffered solutions were as follows: "bicarbonate buffer", 0.1 M sodium bicarbonate and 0.01% $NaN_3$, pH 9.0; "citrate buffer", 0.1 M sodium citrate and 0.01% $NaN_3$, pH 4.5; "deoxycholate buffer", 0.1 M Tris, 0.14 M NaCl, 0.5% sodium deoxycholate, and 0.02% $NaN_3$, pH 8.0 at 25 °C; "digestion buffer", 0.025 M Tris, 2 mM EDTA, and 5 mM cysteine, pH 8.0 at 37 °C; "glycine buffer", 0.1 M glycine and 0.01% $NaN_3$, pH 2.5; "lysis buffer", 0.05 M Tris, 10 mM HEPES, 0.05 M NaCl, 0.165 M sucrose, 5 mM EDTA, and 0.01% $NaN_3$, pH 7.4; "phosphate-buffered saline" (PBS), 0.05 M sodium phosphate, 0.15 M NaCl, and 0.01% $NaN_3$, pH 7.4; "Tris-buffered saline" (TBS), 0.05 M tris(hydroxymethyl)aminomethane (Tris), 0.5 M NaCl, and 0.01% $NaN_3$, pH 7.8 at 4 °C; "Tris buffer", 0.025 M Tris and 0.01% $NaN_3$, pH 8.0 at 4 °C.

conjugated goat anti-(murine IgG); and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. Results were scored visually.

*Fluorescence Microscopy.* The fluorescence microscope consisted of an argon ion laser (Innova 90-3, Coherent Inc., Palo Alto, CA), an inverted optical microscope (Zeiss IM-35, Eastern Microscope CO., Raleigh, NC), and a single-photon-counting photomultiplier (31034A, RCA, Lancaster, PA) interfaced to an IBM PC AT computer, as previously described (Palmer & Thompson, 1989a; Wright et al., 1988).

*Fluorescence Immunoassay for 2.4G2 Binding to J774A.1 Cells.* Aliquots of $10^6$ freshly harvested J774A.1 cells were washed with complete medium and then treated for 20 min with 0.3 mL of 30 $\mu$g/mL fluorescently labeled ("primary" assays) or unlabeled ("secondary" assays) intact or Fab fragments of 2.4G2 or rat IgG in 2 mg/mL BSA/PBS. For secondary assays, cells were then washed twice with medium and treated for 20 min with 0.3 mL of 30 $\mu$g/mL tetramethylrhodamine-labeled rabbit anti-(rat IgG) IgG $(Fab')_2$ in 2 mg/mL BSA/PBS. In both assays, cells were then washed twice with medium and once with PBS and then suspended in 0.2 mL of 10 mg/mL BSA/PBS. All binding and washing steps were carried out at 0–4 °C. The fluorescence arising from antibodies bound to single cells was measured with epifluorescence microscopy (objective, Zeiss, water, 40×, 0.75 N.A.; laser wavelength and power, 514.5 nm and 5.0 $\mu$W). The illuminated area was of radius $\sim$50 $\mu$m, and an image–plane aperture limited fluorescence detection to the area of a single cell.

*Substrate-Supported Planar Membranes.* Following an adaptation of a previously published method (Young et al., 1983), J774A.1 cells [(1-6) × $10^8$, $10^7$ cells/mL] were suspended in lysis buffer containing 1 mM phenylmethanesulfonyl fluoride and disrupted by two cycles of nitrogen cavitation (Model 4639, Parr Instrument Co., Moline, IL) at 500 psi for 30 min on ice. The homogenate was centrifuged at 1000*g* for 10 min and then twice at 20000*g* for 20 min to remove debris (4 °C). Membrane fragments were pelleted by centrifugation at 100000*g* for 1 h, dissolved at the equivalent of $10^8$ cells/mL in deoxycholate buffer, and dialyzed against four 1-L volumes of PBS to produce a solution of protein-containing liposomes. Liposomes were clarified immediately before use by centrifuging twice at 17000*g* for 5 min. In some preparations, 1-acyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]phosphatidylethanolamine (NBD-PE, Avanti Polar Lipids, Birmingham, AL) was added to detergent-solubilized membrane fragments at $\sim$5 mol % before dialysis.

Synthetic vesicles were prepared by a modification of a previously published procedure (Young et al., 1983). A mixture of egg phosphatidylcholine and cholesterol (Sigma) at 6:1 (w/w) in chloroform/methanol (2:1) with 2 mol % NBD-PE was dried under nitrogen, twice resuspended and dried in diethyl ether, and then resuspended at 0.35 mg/mL in lysis buffer that contained 20 mM octyl glucopyranoside (Sigma). The solution was bath sonicated for 2 min and then dialyzed for 36 h at 4 °C against three 1-L volumes of lysis buffer.

Fused silica substrates (1 in. × 1 in. × 1 mm; Quartz Scientific, Fairport Harbor, OH) and glass microscope slides were cleaned with Linbro 7X detergent (Flow Laboratories) as previously described (Thompson et al., 1984b). Immediately before use, slides and substrates were placed in an argon ion plasma cleaner (PDC-3XG, Harrick Scientific Corp., Ossining, NY) for 15 min and then mounted together with a spacer of thickness $\sim$100 $\mu$m. A 70-$\mu$L volume of liposomes prepared from J774A.1 cells or from synthetic lipids was then passed between the substrates and slides and allowed to adsorb for 30 min. Residual liposomes were washed from the inner volume of the sandwich assembly with 5 mL of PBS.

*Lateral Mobility of Fluorescent Lipids in Planar Membranes.* Substrate-supported planar membranes containing NBD-PE and constructed from natural membrane fragments and from synthetic lipid vesicles were observed with epifluorescence microscopy, and the lateral mobility of the incorporated NBD-PE was examined with pattern fluorescence photobleaching recovery (Smith & McConnell, 1978) with the parameters previously described (Wright et al., 1988).

*Interaction of 2.4G2 Fab Fragments and Polyclonal Murine IgG with moFc$\gamma$RII-Containing Planar Membranes.* Substrate-supported planar membranes (without NBD-PE) were treated with 65 $\mu$L of 10 mg/mL BSA/PBS for 30 min and then 250 $\mu$L of various antibody solutions in 5 mg/mL BSA/PBS for 15 min. Antibody solutions contained R-(2.4G2 Fab), R-(rat IgG Fab), murine IgG, and murine IgG $(Fab')_2$ as noted. The fluorescence arising from labeled Fab fragments bound to planar membranes was measured with TIR fluorescence microscopy (Axelrod et al., 1984). Parameters were as follows: laser wavelength and power, 514.5 nm and 50 $\mu$W; fused silica prism, 1 $cm^3$; incidence angle $\alpha$, 75°; elliptically shaped illumination, 20 $\mu$m × 200 $\mu$m; objective (Nikon), air, 10×, 0.25 N.A.; focal length of auxiliary lens, 80 mm; evanescent wave depth, $\sim$700 Å. Experimentally obtained equilibrium binding curves were fit to theoretical forms using the iterative Gauss–Newton nonlinear curve-fitting routine in ASYST software (Macmillan Software Co., Macmillan Publishing Co., New York, NY).

*Solution Concentration of Fab Fragments.* The concentrations of labeled Fab fragments that remained in solution after equilibration with the supported planar membranes were measured with epifluorescence microscopy by focusing the laser beam through auxiliary lenses and a 40× objective (see above) to a small spot in the solution approximately 50 $\mu$m from the two adjacent membrane-coated surfaces. Fluorescence collection was restricted to the region of the focused spot by a pinhole of radius 50 $\mu$m placed at an intermediate image plane. Previous work has shown that these optics produce a beam radius at focus of 0.73 ± 0.09 $\mu$m and that fluorescence collection is limited to a depth of 2.7 ± 0.1 $\mu$m (Palmer & Thompson, 1989b).

## Results and Discussion

The previously reported result that 2.4G2 cells (a rat–mouse hybridoma) secrete two antibodies, one that binds to murine macrophage cell surfaces and has rat $\kappa$ light chains and one that does not bind to macrophages and has murine light chains derived from the P3U1 fusion partner (Unkeless, 1979; Lamers et al., 1984; Phillips & Parker, 1984), was confirmed in the present work. Two types of antibodies were copurified from 2.4G2 cell supernatants by goat anti-(rat IgG) affinity chromatography as judged by SDS–PAGE under reducing and nonreducing conditions. The two 2.4G2 antibodies could be separated by anti-(rat $\kappa$ light chain) (MAR18.5) affinity chromatography. The 2.4G2 antibodies that were bound (2.4G2+) and were not bound (2.4G2–) to MAR18.5-coupled Sepharose had light chains of apparent molecular weights 23K and 25K, respectively. Enzyme-linked immunoassay confirmed that only the 2.4G2+ antibodies contained rat $\kappa$ light chains. In addition, different 2.4G2 cell populations secreted primarily 2.4G2+, primarily 2.4G2–, or a mixture of 2.4G2+ and 2.4G2– antibodies. Of utility was the observation that cell culture supernatants could be screened by SDS–PAGE under

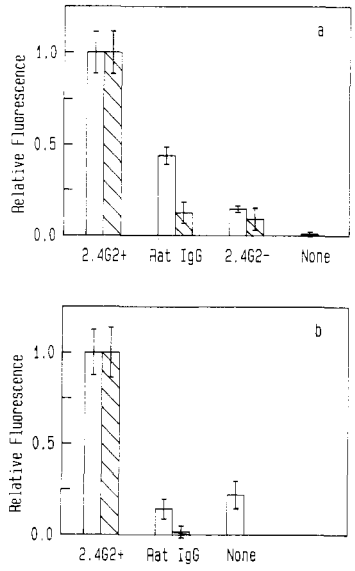


FIGURE 1: Fluorescence immunoassay for 2.4G2 binding to macrophage-related cell surfaces. Shown is the average measured fluorescence from single J774A.1 cells treated with unlabeled, intact IgG antibodies followed by labeled rabbit anti-(rat IgG) IgG $(Fab')_2$ (panel a, open bars), labeled, intact IgG antibodies (panel a, hatched bars), unlabeled Fab fragments followed by labeled rabbit anti-(rat IgG) IgG $(Fab')_2$ (panel b, open bars), and labeled Fab fragments (panel b, hatched bars). The measured fluorescence has been corrected for the background fluorescence from untreated cells (all data) and for the relative fluorescence yields of different labeled antibodies (hatched bars). Shown are the means and standard errors of the mean for 30–36 cells per antibody combination.

reducing conditions for the presence of 2.4G2+ light chains.

Specificity of the purified 2.4G2 antibodies for J774A.1 cells was confirmed by two immunofluorescence assays. In the first assay, J774A.1 cells, which have membrane-bound moFc$_\gamma$RII, were treated with unlabeled 2.4G2+, 2.4G2–, or rat IgG antibodies and then with fluorescently labeled rabbit anti-(rat IgG) IgG $(Fab')_2$ fragments. The average single-cell fluorescence was significantly higher for cells treated with 2.4G2+ antibodies rather than 2.4G2–, rat IgG, or no primary antibody (Figure 1a). To rule out the possibility that this result arose from heterogeneous affinities of the second-step antibody for the different primary antibodies, another assay was done in which J744A.1 cells were treated with either fluorescently labeled 2.4G2+, 2.4G2–, or rat IgG antibodies of similar fluorescence yields. In this assay, the average single-cell fluorescence, normalized by the relative fluorescence of the different labeled antibodies as measured spectrofluorometrically, was 10-fold higher for cells treated with 2.4G2+ (Figure 1a). The nonzero fluorescence measured for cells treated with 2.4G2– or rat IgG could result from Fc-mediated binding to moFc$_\gamma$RII or other IgG Fc receptors on the macrophage cell surface.

2.4G2+ Fab fragments were produced by papain digestion and purified by MAR18.5 affinity chromatography as judged by SDS–PAGE under reducing and nonreducing conditions. As shown in Figure 1b, substantially more fluorescently labeled anti-(rat IgG) IgG $(Fab')_2$ bound to cells treated with 2.4G2+ Fab than to those treated with rat IgG Fab or with no unlabeled Fab, and fluorescently labeled 2.4G2+ Fab bound to J774A.1 cells whereas fluorescently labeled rat IgG Fab did not appreciably bind. These results confirmed that 2.4G2+ but not 2.4G2– or rat IgG antibodies specifically bound to J774A.1 cells through their antigen binding regions.

Substrate-supported planar membranes containing moFc$_\gamma$RII were prepared from J774A.1 cells by a modification

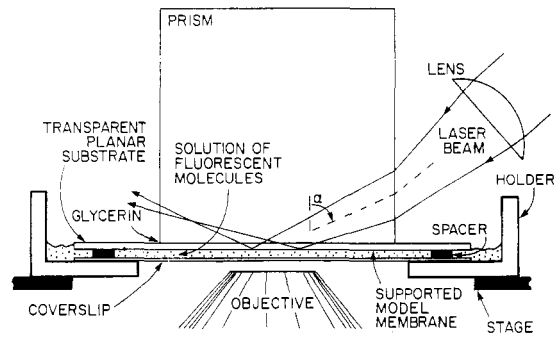


FIGURE 2: Apparatus for TIR fluorescence microscopy. A fused silica cubic prism is optically coupled to the membrane-supporting substrate with glycerin. A laser beam is roughly focused and totally internally reflected at the membrane/solution interface. Surface-associated fluorescence excited by the evanescent field is collected by a microscope objective.

of previously published procedures for isolating J774A.1 membrane fragments (Young et al., 1983; Klempner et al., 1980) and for depositing membrane fragments of other cell types on planar substrates (Watts et al., 1984). The properties of the planar membranes constructed from J774A.1 cells were investigated by labeling the membranes with the fluorescent lipid NBD-PE. Epifluorescence microscopy indicated that the substrate was uniformly coated with fluorescent lipids within optical resolution, and pattern fluorescence photobleaching recovery demonstrated that the lateral mobility of the fluorescent lipids was very low ($\leq 5 \times 10^{-12}$ cm$^2$/s). However, fluorescent lipid mobility was measured to be $\sim 2 \times 10^{-8}$ cm$^2$/s in substrate-supported planar membranes formed from synthetic lipid vesicles; this result is consistent with previous reports that fluorescent lipids in supported planar membranes constructed from lipid vesicles with and without reconstituted proteins are laterally mobile with diffusion coefficients $\sim 3.5 \times 10^{-8}$ cm$^2$/s (Brian & McConnell, 1984; Watts et al., 1984). The lack of appreciable lateral mobility of fluorescent lipids in planar membranes constructed from J774A.1 membrane fragments suggests that the fragments do not completely fuse at the substrate or that long-range lateral mobility is inhibited by the chemical composition.

The specific binding of fluorescently labeled 2.4G2+ Fab fragments to macrophage-derived substrate-supported planar membranes was examined with TIR fluorescence microscopy (Figure 2). The fluorescence excited by the evanescent wave ($F_1$) as a function of the applied concentration of labeled Fab fragments, denoted by $[R\text{-}(2.4G2+\ Fab)]_a$ and $[R\text{-}(rat\ IgG\ Fab)]_a$, is shown in Figure 3a. The measured fluorescence increased with $[R\text{-}(2.4G2+\ Fab)]_a$ to a saturation level whereas very little surface-associated fluorescence was detected for the same range of $[R\text{-}(rat\ IgG\ Fab)]_a$. As shown in Figure 3b, fluorescence ($F_2$) arising from labeled Fab fragments in solution, denoted by $[R\text{-}(2.4G2+\ Fab)]_f$ and $[R\text{-}(rat\ IgG\ Fab)]_f$, was detected for all but the two lowest applied concentrations of R-(Fab). The solution fluorescence was linear with $[R\text{-}(rat\ IgG\ Fab)]_a$ and for the higher values of $[R\text{-}(2.4G2+\ Fab)]_a$ and was used as a calibration for the lower R-(2.4G2+ Fab) solution concentrations.

In the simplest case, the normalized surface-associated fluorescence ($F_1$, Figure 3a) measured as a function of the solution concentration of labeled 2.4G2+ Fab fragments, [A], should be of the form

$$F_1([A]) = \frac{K_1[A]}{1 + K_1[A]} \qquad (1)$$

where $K_1$ is the equilibrium constant for the association of

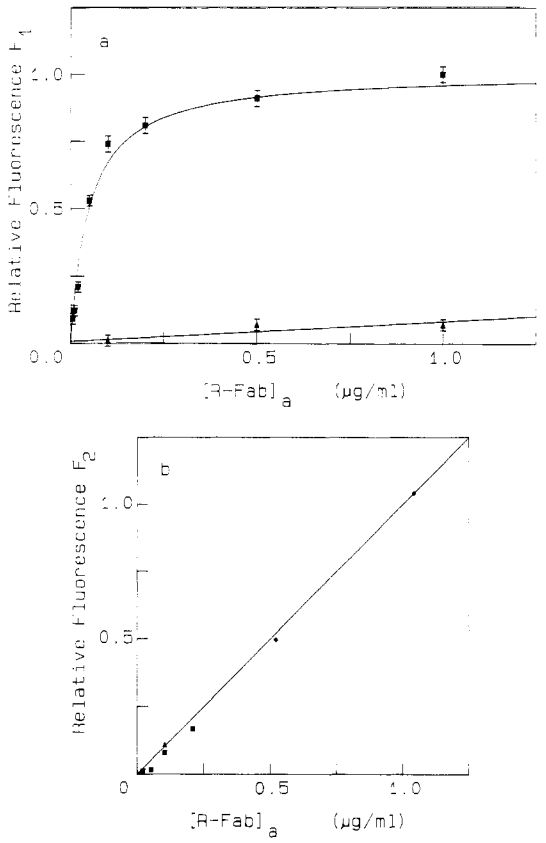


FIGURE 3: Association of 2.4G2+ Fab fragments with supported planar membranes. (a) The fluorescence ($F_1$) excited by the evanescent field and arising from fluorescently labeled 2.4G2+ Fab fragments bound to substrate-supported planar membranes constructed from J774A.1 membrane fragments increases to saturation with the applied concentration of R-(2.4G2+ Fab) (■). Very little fluorescence was measured for several applied concentrations of fluorescently labeled rat IgG Fab fragments (▲). (b) The fluorescence ($F_2$) excited by a laser beam focused to a small spot in the solution adjacent to supported planar membranes and arising from (■) R-(2.4G2+ Fab), (▲) R-(rat IgG Fab), and (♦) both R-(2.4G2+ Fab) and R-(rat IgG Fab) (points overlapping) is proportional to the solution concentration. The standard errors in the mean are smaller than the size of the plotted points. In (a) and (b), the measured fluorescence has been corrected for the relative fluorescence yields of different labeled antibodies.

2.4G2+ Fab with moFc$_\gamma$RII. Consistent with Eq 1, a plot of $F_1/(1 - F_1)$ was linear with zero intercept, and the slope given by a least-squares analysis yielded an association constant of $K_1 = (9.6 \pm 0.4) \times 10^8$ M$^{-1}$ for the binding of 2.4G2+ Fab fragments to moFc$_\gamma$RII in planar membranes. This measured value is consistent with that previously reported for the association of radioactively labeled 2.4G2+ Fab fragments with J774A.1 cell surfaces (Mellman & Unkeless, 1980).

The observation that very little surface-associated fluorescence is detected for planar membranes treated with [R-(rat IgG Fab)]$_a$ = 1 μg/mL, the saturating value of [R-(2.4G2+ Fab)]$_a$, indicates that nearly all of the measured fluorescence $F_1$ arises from R-(2.4G2+ Fab) fragments that are bound to the surface rather than in solution but within the evanescent wave depth. This means that the density of membrane-bound R-(2.4G2+ Fab) is much greater than (1 μg/mL)(700 Å) = 1 molecule/μm$^2$. In addition, the nonlinearity of the plot in Figure 3b for values of [R-(2.4G2+ Fab)]$_a$ <200 ng/mL indicates that the planar membranes significantly deplete the solution of R-(2.4G2+ Fab). The average amount by which the applied concentrations were depleted was 22 ± 2 ng/mL. This value, together with the known sample volume (250 μL) and the total planar membrane surface area (2 in.$^2$), implies that the density of bound R-(2.4G2+ Fab) is ≤50 molecules/μm$^2$. Assuming that the surface density of moFc$_\gamma$RII on J774A.1 cells is approximately 500 000 copies (Mellman & Unkeless, 1980) distributed over 400 μm$^2$ (Petty et al., 1981), then the receptors are diluted ~25-fold in the substrate-supported planar membranes.

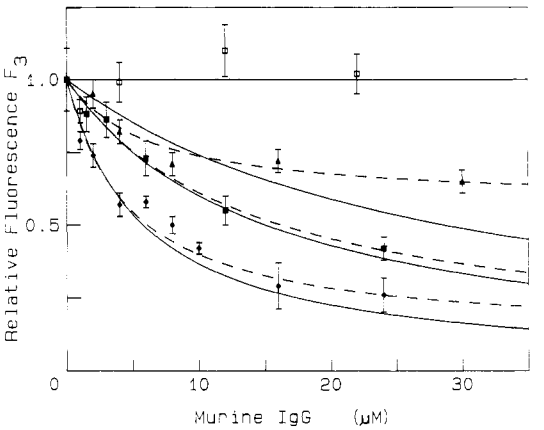


FIGURE 4: Association of polyclonal murine IgG with supported planar membranes. The normalized fluorescence obtained with evanescent field excitation and arising from R-(2.4G2+ Fab) bound to planar membranes decreases as a function of the concentration of unlabeled murine IgG for applied concentrations of R-(2.4G2+ Fab) equal to 50 ng/mL (♦), 100 ng/mL (■), and 200 ng/mL (▲), but remains constant as a function of the concentration of unlabeled polyclonal murine IgG (Fab′)$_2$ for [R-(2.4G2+)]$_a$ = 50 ng/mL (□). Solid and dashed lines indicate the best fits of the experimental data to eq 3 and 5, respectively.

The Fc binding activity of moFc$_\gamma$RII in the supported planar membranes was tested by measuring the fluorescence of surface-bound R-(2.4G2+ Fab) fragments in the presence of unlabeled polyclonal murine IgG. As shown in Figure 4, murine IgG inhibited the binding of labeled 2.4G2+ Fab fragments to supported planar membranes whereas murine IgG (Fab′)$_2$ did not inhibit R-(2.4G2+ Fab) binding. In addition, the degree of inhibition was dependent on the concentration of R-(2.4G2+ Fab).

A possible mechanism for the inhibition shown in Figure 4 is one in which murine IgG completely blocks the interaction between 2.4G2+ Fab fragments and moFc$_\gamma$RII. In this case, the measured fluorescence, $I_3$, is proportional to the surface concentration of bound 2.4G2+ Fab fragments:

$$I_3([M]) \propto \frac{K_1[A]}{1 + K_1[A] + K_2[M]} \quad (2)$$

where [M] is the concentration of murine IgG, $K_2$ is the association constant for murine IgG with moFc$_\gamma$RII, and $K_1$ and [A] are defined above. The inverse of the measured fluorescence, normalized to its value in the absence of murine IgG, is given by

$$F_3^{-1}([M]) = \frac{I_3(0)}{I_3([M])} = 1 + \frac{K_2[M]}{1 + K_1[A]} \quad (3)$$

However, the nature of the 2.4G2+ epitope is not well understood, and a more complex but perhaps more accurate mechanism is one in which IgG does not inhibit but does reduce the affinity of the interaction between 2.4G2+ Fab and moFc$_\gamma$RII. In this mechanism, $I_3$ is proportional to the sum of surface-bound (2.4G2+ Fab)–moFc$_\gamma$RII and (2.4G2+ Fab)–IgG–moFc$_\gamma$RII complexes and is given by

$$I_3([M]) \propto \frac{K_1[A] + K_2[M]K_3[A]}{1 + K_1[A] + K_2[M](1 + K_3[A])} \quad (4)$$

and the inverse of the normalized fluorescence equals

$$F_3^{-1}([M]) = \frac{I_3(0)}{I_3([M])} = \frac{1 + K_2[M](1 + K_3[A])/(1 + K_1[A])}{1 + (K_3/K_1)K_2[M]} \quad (5)$$

where $K_3$ is the association constant of 2.4G2+ Fab fragments with the IgG–moFc$_\gamma$RII complex and is less than $K_1$. A key difference between the mechanisms described by eq 2 and 4 is that $I_3$ approaches zero at large concentrations of IgG in the first mechanism but approaches a nonzero value given by eq 1 with $K_1 = K_3$ in the second mechanism. When $K_3 = 0$, the mechanisms are equivalent, and eq 4 reduces to eq 2.

The three curves in Figure 4, measured for different solution concentrations of R-(2.4G2+ Fab), were fit to the functional form of eq 3 with $K_2$ as a free parameter and to the form of eq 5 with $K_2$ and $K_3$ as free parameters. In both analyses, $K_1$ and [A] were set equal to their experimentally determined values. The fits to eq 5 appear more accurate, but the increased accuracy of the fit may not be statistically significant because the second mechanism contains an additional free parameter. The values of $K_2$ obtained from the fits to eq 3 ranged from $1.7 \times 10^5$ to $3.5 \times 10^5$ M$^{-1}$, the values of $K_2$ obtained from the fits to eq 5 ranged from $2.1 \times 10^5$ to $4.4 \times 10^5$ M$^{-1}$, and the values of $K_3$ obtained from the fits to eq 5 ranged from $0.3 \times 10^8$ to $2.1 \times 10^8$ M$^{-1}$. Both mechanisms imply that the IgG–moFc$_\gamma$RII interaction is described by an association constant in the range of $(1–5) \times 10^5$ M$^{-1}$; this value is slightly lower than that obtained for the binding of murine IgG to macrophage cell surfaces as measured by competitive assays with radioactively labeled ligands under nonequilibrium conditions (Dower et al., 1981a,b; Burton, 1985). The best-fit values of $K_3$ compared to the measured value of $K_1$ imply that murine IgG reduces the affinity of 2.4G2+ Fab fragments for moFcR$_\gamma$II by a factor of at least 5.

## Conclusion

The application of TIR fluorescence microscopy to specific ligand–receptor interactions has been suggested for a number of years (Burghardt & Axelrod, 1981; Axelrod et al., 1984; Thompson et al., 1988). A key to this spectroscopic approach has been the development of substrate-supported planar membranes that contain functionally reconstituted membrane receptors (McConnell et al., 1986). We have described in this work the adaptation of a relatively simple procedure for constructing substrate-supported planar membranes from isolated cell membrane fragments (Watts et al., 1984) to macrophage membranes containing moFc$_\gamma$RII. As shown, the specific interaction of anti-moFc$_\gamma$RII receptor antibodies and of murine IgG with the reconstituted moFc$_\gamma$RII could be quantitatively investigated with TIR fluorescence microscopy.

Theoretically, the range of equilibrium constants that can be measured by TIR fluorescence microscopy is lower-limited by the requirement that a significant fraction of the measured fluorescence arises from surface-bound fluorophores rather than fluorophores in solution but excited by the evanescent illumination. This requirement means that the equilibrium constant must be greater than $\sim 10d/N$ (Thompson et al., 1988), where $d$ is the evanescent wave depth and $N$ is the total surface site density. For the supported planar membranes described in this work in which the density of moFc$_\gamma$RII is very low, equilibrium constants less than $\sim 10^7$ M$^{-1}$ would be difficult to measure. Thus, although the association of anti-moFc$_\gamma$RII Fab fragments with the planar membranes was characterized by a direct assay, moFc$_\gamma$RII Fc binding activity was demonstrated with a competitive assay. However, the moFc$\gamma$RII surface site density might be increased by as much as a factor of 100 if the receptors were purified and then reconstituted in supported planar membranes, and the evanescent wave depth might be decreased 2–4-fold by using substrates with a higher refractive index (Axelrod et al., 1984). This approach would allow direct observation of fluorescently labeled IgG bound to moFc$_\gamma$RII under equilibrium conditions. Potential applications would then include measuring the kinetics of IgG–moFc$_\gamma$RII interactions by combining TIR illumination with fluorescence photobleaching recovery or correlation spectroscopy (Burghardt et al., 1981; Thompson & Axelrod, 1983), investigating the possible clustering of IgG–moFc$_\gamma$RII complexes by scanning fluorescence correlation spectroscopy (Palmer & Thompson, 1989a), and examining possible changes in moFc$_\gamma$RII conformation as characterized by fluorescence energy transfer (Watts et al., 1986) in conjunction with measurements of the orientation distribution of the reconstituted moFc$_\gamma$RII (Thompson et al., 1984a).

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# Protective Effect of Lipidic Surfaces against Pressure-Induced Conformational Changes of Poly(L-lysine)†

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ABSTRACT: Poly(L-lysine) bound to phosphatidylglycerol or phosphatidic acid bilayers was submitted to hydrostatic pressure in a diamond anvil cell to investigate whether the lipidic surfaces can protect the polypeptide against pressure-induced conformational transformations. The amide I region of the infrared spectrum of dimyristoylphosphatidic acid bound polylysine shows that most of the polypeptide retains its $\beta$-sheet structure up to 19 kbar, while it is known to convert entirely to $\alpha$-helix at $\sim$2 kbar in the absence of the lipid [Carrier, D., Mantsch, H. H., & Wong, P. T. T. (1989) *Biopolymers* (in press)]. The simultaneous binding of the polypeptidic molecules to two opposing bilayers appears to be required in order to preserve the $\beta$-sheet structure at pressures over $\sim$9 kbar: a small proportion of the polypeptide, most likely the molecules at the surface of the aggregated bilayers, was found to convert to unordered and eventually to $\alpha$-helical conformations in the pressure range 9–19 kbar. The decrease from 1612 to 1606 $cm^{-1}$ of the frequency of the major $\beta$-sheet component of the infrared amide I band as the pressure is raised to 6 kbar indicates a strengthening of the interchain hydrogen bonds. The high-pressure infrared spectra of polylysine bound to dimyristoyl- and dipalmitoylphosphatidylglycerol show that the polypeptide remains $\alpha$-helical up to $\sim$12 kbar, though the changes in the bandshape indicate an increase in hydrogen bond strength. The formation of a small amount of $\beta$-sheet was observed during decompression and is attributed to the effect of dehydration on the polypeptidic molecules located at the surface of the aggregates. This study suggests that lipidic surfaces could be used as cocatalysts, to control and protect the active conformation of enzymatic proteins for operation at elevated hydrostatic pressure.

Poly(L-lysine) is frequently used as a model protein because of its ability to adopt three different conformations: $\alpha$-helix, $\beta$-sheet, and an unordered structure which has been proposed to actually consist of extended helices (Krimm & Tiffany, 1974; Paterlini et al., 1986). A high pH value (>10.5) is required for the formation of the first two conformers in aqueous solution; nearly pure $\alpha$-helix or $\beta$-sheet conformations can be obtained if the temperature is maintained below 4 °C or raised above 50 °C, respectively. The conformation of polylysine can also be modulated by other means. At neutral pH, it forms $\alpha$-helices upon binding to phosphatidylglycerol bilayers (Carrier & Pézolet, 1984) while binding to phosphatidic acid bilayers is accompanied by conversion to $\beta$-sheets (Laroche et al., 1988). More recently, hydrostatic pressure has been shown to promote the formation of $\alpha$-helices from unordered as well as from $\beta$-sheet polylysine in aqueous solution (Carrier et al., 1989). The purpose of the present infrared spectroscopic study is to investigate the effect of high pressure on the polypeptide already bound to dimyristoylphosphatidic acid (DMPA),[1] DPPG, or DMPG. In other words, we want to establish whether the surface of a lipidic bilayer can protect the polypeptide against pressure-induced denaturation.

## Experimental Procedures

Poly(L-lysine) hydrobromide of molecular weight 271 000 and the sodium salt of dimyristoyl-L-$\alpha$-phosphatidic acid

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[1] Abbreviations: DMPA, dimyristoylphosphatidic acid; DMPG, dimyristoylphosphatidylglycerol; DPPG, dipalmitoylphosphatidylglycerol; FT-IR, Fourier-transform infrared.

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