Lipid Monolayer-coated Solid Surfaces Do Not Perturb the Lateral Motion and Distribution of C3b Receptors on Neutrophils

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ABSTRACT We have used epifluorescence and photobleaching techniques to study the lateral distribution and motion of fluorescein-conjugated Fab fragments of anti-C3b receptor antibody bound to human neutrophils when the cells rest on various solid supports (microscope slides or cover slips). Supports composed of quartz, glass, or alkylated glass induce cellular adhesion, spreading, and an extensive lateral redistribution of C3b receptors (but not HLA antigens). The neutrophil C3b receptors become patchy, and the patches apparently undergo nonrandom translational motion. Many patches are found on the upper surfaces of the cells removed from the region of cell membrane-glass contact. In contrast, neutrophils supported by lipid monolayer-coated glass do not adhere or spread, and the C3b receptor remains uniform and diffuses freely ($D \cong 2 \times 10^{-10}$ cm^2/s).

Previous studies of the lateral motion and distribution of the complement (C3b) receptor on peripheral blood leukocytes using fluorescent antireceptor antibody and antireceptor antibody fragments have indicated that these receptors are highly clustered (3, 9). Indirect measurements have also suggested that the C3b receptor is immobile on normal resting macrophages (4, 7, 8). Because cell surface receptors usually have a diffuse distribution in the absence of receptor-ligand interactions, we suspected that the clustered distribution of the C3b receptors might be a consequence of cell triggering. Concurrent studies in this laboratory have recently shown that macrophages (5), basophils (13), and neutrophils (D. G. Hafeman, M. Seul, and H. M. McConnell, unpublished observation) do not bind to lipid monolayer-coated alkylated glass surfaces in the absence of specific antibody-mediated monolayer-cell membrane interaction. We therefore undertook a study to determine whether interaction of cells with solid supports could influence the distribution and motion of the C3b receptor on human neutrophils. We find that the distribution and motion of the C3b receptor is strongly affected by the nature of the substrate on which the cells are examined. In contrast, HLA antigens are relatively unaffected by substrate contact.

MATERIALS AND METHODS

Preparation of Cells

Whole blood was drawn from healthy human donors. Fibrin and platelets were removed by gentle swirling of the blood with 3-mm-diameter solid glass beads (one bead/ml) for 10 min. Blood was diluted with 1 vol of 0.85% NaCl. An aliquot of 6% dextran in 0.85% NaCl (T-500; Pharmacia Fine Chemicals, Piscataway, NJ) was added to give a final dextran concentration of 0.6%. The erythrocytes were allowed to sediment at 1 *g* for 40 min at 24°C. The supernatant was layered onto a cushion of Ficoll-Hypaque (Pharmacia) and granulocytes and mononuclear leukocytes were separated by centrifugation at 400 *g* for 40 min. Each cell fraction was washed twice in Ca^{++}- and Mg^{++}-free Earle's balanced salt solution with 0.2% gelatin and buffered with 25 mM HEPES (pH 7.4). All cells were resuspended at 5×10^6/ml in cell buffer (CB; 2.0 mM $CaCl_2$, 1.5 mM $MgCl_2$, 5.4 mM KCl, 1 mM NaH_2PO_4, 5.6 mM glucose, 120 mM NaCl, 25 mM HEPES, and 0.2% bovine serum albumin [fatty acid poor; Calbiochem-Behring Corp., San Diego, CA] pH 7.4). Cell viability assessed by trypan blue exclusion was in all cases >95%.

Antibodies

Monoclonal anti-HLA, A, B, C, (W6/32) antibody was the generous gift of Dr. Peter Parham (Department of Structural Biology, Stanford University Medical Center) and has been described elsewhere (2). The Fab fragment was prepared by papain digestion (1.7 mg/ml antibody, 0.29 mg/ml papain, 2 mM cysteine, 4 mM EDTA, 100 mM acetate buffer pH 5.5) at 37°C for 9 h. The reaction was quenched by addition of iodoacetamide to a final concentration of 5 mM and incubation at 37°C for 30 min. The digestion mixture was adjusted to neutral pH and passed through a protein A-Sepharose (Pharmacia) affinity column to remove any Fc-containing material. Analysis by SDS PAGE showed no detectable intact heavy chain.

The $F(ab')_2$ fragment of a rabbit anti-C3b receptor antibody was prepared as described previously (9). The Fab' fragment was prepared by reduction with 20 mM cysteine at 37°C for 4 h followed by alkylation with excess iodoacetamide for 30 min at 37°C.

Fluorescein conjugates of the antibody fragments were prepared by reaction with excess fluorescein isothiocyanate (FITC) (overnight at 4°C and pH 9.0) followed by purification by Sephadex G-25 gel chromatography. The conjugates were kept at 4°C in the presence of 0.02% sodium azide. Aliquots for use in experiments were centrifuged at 100,000 *g* for 20 min periodically. Protein concentrations were of the order of 0.2–1 mg/ml.

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Immunofluorescent Labeling

20 μl of the cell suspension (5×10^6/ml) and 3 μl of the fluorescein-conjugated Fab or Fab' fragments were incubated for 15 min on ice. Cells were washed three times with 1 ml of CB by centrifugation at 1,000 *g* for 2 min at 4°C. Cells were resuspended in 30 μl of cold CB, transferred to the appropriate substrate, and examined and/or photobleached.

Fluorescence Photomicrography

The apparatus used for the photography of weakly fluorescent cells has been described previously (11). A Zeiss Photomicroscope III was fitted with an image intensifier (Ni-Tec, Skokie, IL), which was in turn attached to a Nikon EL-2 35-mm camera. Kodak 2475 recording film was exposed and processed at ASA 3200. The fluorescence was excited with the 488-nm line of an argon ion laser (Spectra Physics no. 164-05, Sunnyvale, CA) at a light intensity of ~1 $\mu W/\mu m^2$.

Diffusion Measurements

The techniques and instrumentation used for measuring lateral diffusion of membrane-bound fluorescent antibody fragments were similar to those described by Petty et al. (9). However, in the present work a 63× (N.A. of 1.2) water-immersion Plan-Neofluar objective (Zeiss) was used. The fluorescence recovery after a brief intense bleaching was measured using photon counting methods as described previously (9). Typically 100 data points of 2,000 photons each were taken at 1-s intervals. Data were analyzed by computer least squares fit to a single exponential, yielding the time constant and amplitude of recovery.

Monolayer Membranes

Monolayer membranes composed of dipalmitoylphosphatidylcholine (DPPC) were deposited at room temperature from an air-water interface (pressure, 30 dyn/cm) onto an alkylated glass cover slip as described previously (5, 12).

RESULTS

Fig. 1 shows image-intensified fluorescence photomicrographs of neutrophils labeled with FITC-Fab'-anti-C3b receptor. When the labeled cells are placed on quartz slides at room temperature the C3b receptor is initially uniform (Fig. 1 *a*). Subsequently, the cells spread and there is an extensive redistribution and patching of the C3b receptors (Fig. 1 *b*). Similar results are obtained on glass and silanized glass surfaces (the alkylated microscope cover slips before deposition of a phospholipid monolayer), except that these cellular responses occurred more slowly on the silanized glass surfaces. In contrast, the cells on monolayer-coated cover slips do not spread even at long times (31 min) and retain their initial uniform distribution of C3b receptor–associated fluorescence (Fig. 1 *c*).

The surface receptor redistribution that was induced by cell-substrate contact is relatively specific for the C3b receptor. Neutrophils similarly labeled with FITC-Fab-anti-HLA (A, B,

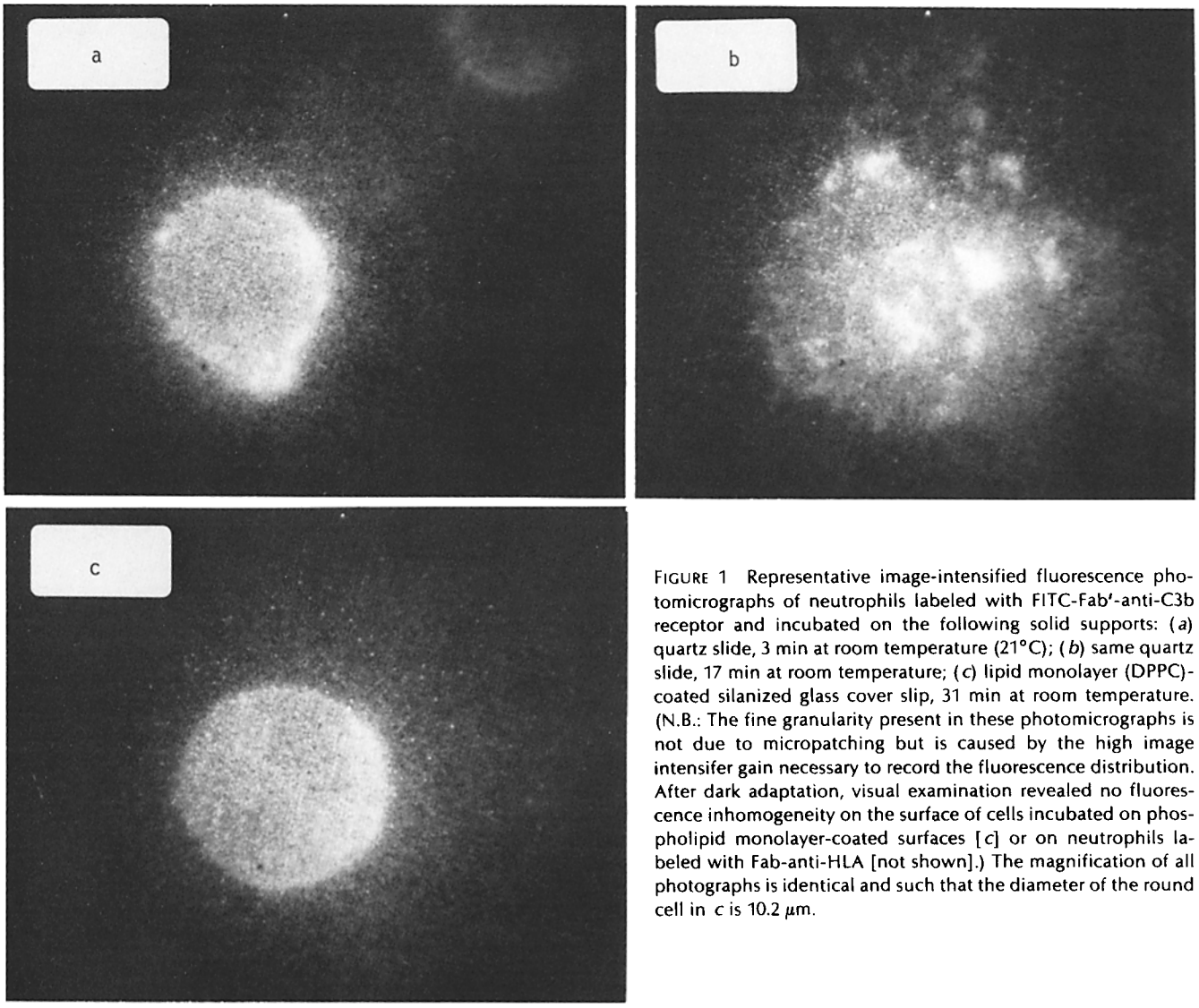

FIGURE 1 Representative image-intensified fluorescence photomicrographs of neutrophils labeled with FITC-Fab'-anti-C3b receptor and incubated on the following solid supports: (*a*) quartz slide, 3 min at room temperature (21°C); (*b*) same quartz slide, 17 min at room temperature; (*c*) lipid monolayer (DPPC)-coated silanized glass cover slip, 31 min at room temperature. (N.B.: The fine granularity present in these photomicrographs is not due to micropatching but is caused by the high image intensifer gain necessary to record the fluorescence distribution. After dark adaptation, visual examination revealed no fluorescence inhomogeneity on the surface of cells incubated on phospholipid monolayer-coated surfaces [*c*] or on neutrophils labeled with Fab-anti-HLA [not shown].) The magnification of all photographs is identical and such that the diameter of the round cell in *c* is 10.2 μm.

C) display a uniform fluorescence distribution even on cells that have spread extensively on the substrate.

Lateral diffusion measurements on adherent neutrophils by the fluorescence pattern photobleaching technique yield erratic recovery curves when the fluorescence is associated with the C3b receptor (Fig. 2*a*). In contrast, recovery curves for fluorescence associated with HLA (A, B, C) on similar adherent cells are regular and typical for mobile membrane proteins (Fig. 2*b*). This erratic "recovery" associated with the C3b receptor is thought to be due to active (nondiffusive) motion of clusters of C3b receptors. Similar erratic recovery curves have been seen for other antigens on other cells.[1]

Neutrophils supported by lipid monolayer–coated cover slips interact with this solid surface so weakly that thermal and other cellular motions prevent accurate photobleach recovery measurements. Visual observation of the decay of a photobleach stripe pattern of labeled C3b receptors yields estimated diffusion coefficients of the order of 2×10^{-10} cm^2/s, consistent with measurements obtained from a small fraction of adherent cells (see legend to Fig. 2).

In numerous unpublished experiments in this laboratory we have used a technique devised by Dr. Martin Schwartz (personal communication) to establish whether the fluorescence from a given cell is or is not associated with the outer surface of the plasma membrane. The technique involves quickly examining the cells after replacement of the external medium with an isotonic aqueous solution of 10 mM cupric sulfate, pH 5. The cupric ion quenches the fluorescence of externally accessible molecules. Such measurements indicate that FITC-Fab′-anti-C3b receptor is on the outer surface of the plasma membrane, since the fluorescence of the cells is completely quenched. Control experiments with cells that have internalized FITC-conjugated proteins do not show a substantial quenching of fluorescence under these conditions.

The patchy fluorescence of the FITC-Fab′-anti-C3b receptor antibody was clearly not localized to the region of contact between the adherent cells and the solid support. This was established by adjustment of the focal plane of the microscope objective lens, which has a narrow depth of focus (~0.7 μm). In many cases patchy fluorescence was clearly located at the upper surface.

DISCUSSION

One important result of the present work is that it is possible to prepare a lipid monolayer surface that is apparently entirely free of nonspecific binding or other interaction with neutrophils. Although this result might have been anticipated from our earlier studies with other cell types, the neutrophils provide a particularly severe test for the absence of nonspecific interactions, since these cells adhere strongly to a wide variety of surfaces (1, 6).

The second significant observation we have made is that the distribution and motion of the C3b receptor can be strongly modified when the neutrophil adheres to various solid surfaces. The C3b receptor distribution becomes nonuniform not only in the region of cell membrane–solid surface contact, but also in regions of the plasma membrane far removed from the solid surface. This is reminiscent of the "global" immobilization of concanavalin A (Con A) receptors on lymphocytes due to localized binding of Con A (10). Obviously, there is some degree of specificity in the redistribution of C3b receptors reported here, since no comparable patching of HLA antigens was observed under identical conditions. This difference is also manifest in the typically smooth, "normal" photobleach recovery curves for FITC-Fab-anti-HLA compared with the typical erratic recovery curves found for FITC-Fab′-anti-C3b receptor. This erratic recovery may reflect cytoskeleton-mediated motion of patches of FITC-Fab′-anti-C3b receptor on the neutrophil surface.

These observations show that cellular interactions with solid supports can affect the distribution and motion of cell surface molecules. Clearly, studies seeking to relate the distribution and motion of cell surface receptors to cell function must be interpreted with caution if simultaneous nonspecific interactions between cells and solid surfaces exist.

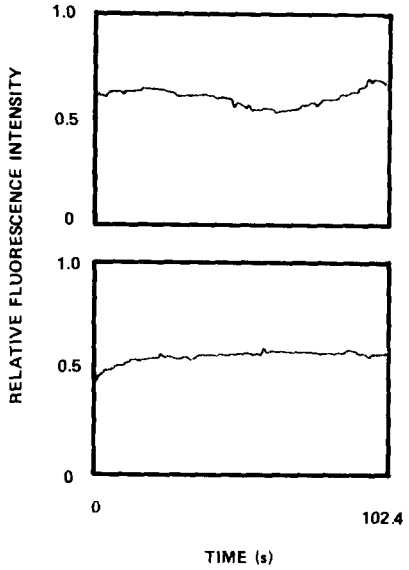

FIGURE 2 Representative photobleach recovery curves for FITC-Fab′-anti-C3b receptor (*top*), and FITC-Fab-anti-HLA (A, B, C) (*bottom*), both on human neutrophils on quartz slides. Diffusion coefficients for HLA (A, B, C) are estimated to be of the order of 5×10^{-10} cm^2/s. Fluorescence recovery for FITC-Fab′-anti-C3b receptor was often too erratic to estimate diffusion coefficients; in a few cases the diffusion coefficient of C3b receptor appeared to be of the order of 2×10^{-10} cm^2/s.

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REFERENCES

1. Boyles, J., and D. F. Bainton. 1979. Changing patterns of plasma membrane-associated filaments during the initial phases of polymorphonuclear leukocyte adherence. *J. Cell Biol.* 82:347–368.
2. Brodsky, F. M., P. Parham, C. J. Barnstable, M. J. Crumpton, and W. F. Bodmer. 1979. Monoclonal antibodies for analysis of the HLA system. *Immunol. Rev.* 47:3–61.
3. Fearon, D. T., I. Kaneko, and G. G. Thompson. 1981. Membrane distribution and adsorptive endocytosis by C3b receptors on human polymorphonuclear leukocytes. *J. Exp. Med.* 153:1615–1628.
4. Griffin, F. M., Jr., and P. J. Mullinax. 1981. Augmentation of macrophage complement function in vitro. III. C3b receptors that promote phagocytosis migrate within the plane of the macrophage plasma membrane. *J. Exp. Med.* 154:291–305.

[1] Howard, F. D., J. A. Ledbetter, D. P. Carter, L. M. Smith, and H. M. McConnell. The lateral mobility and surface distribution of Lyt-1, Lyt-2 and Lyt-3 on mouse thymocytes. Manuscript submitted for publication.

5. Hafeman, D. G., V. von Tscharner, and H. M. McConnell. 1981. Specific antibody-dependent interactions between macrophages and lipid haptens in planar lipid monolayers. *Proc. Natl. Acad. Sci. U. S. A.* 78:4552–4556.
6. Klebanoff, S. J., and R. A. Clark. 1978. The Neutrophil: Function and Clinical Disorders. North-Holland Publishing Co., New York. 75.
7. Michl, J., M. M. Pieczonka, J. C. Unkeless, and S. C. Silverstein. 1979. Effects of immobilized immune complexes on Fc- and complement-receptor function in resident and thioglycollate-elicited mouse peritoneal macrophages. *J. Exp. Med.* 150:607–621.
8. Michl, J., J. C. Unkeless, M. M. Pieczonka, and S. C. Silverstein. 1979. Mechanism of macrophage Fc receptor modulation by immobilized antigen-antibody complexes. *J. Cell Biol.* 83(2, Pt. 2):295 *a* (Abstr.).
9. Petty, H. R., L. M. Smith, D. T. Fearon, and H. M. McConnell. 1980. Lateral distribution and diffusion of the C3b receptor of complement, HLA antigens, and lipid probes in peripheral blood leukocytes. *Proc. Natl. Acad. Sci. U. S. A.* 77:6587–6591.
10. Schlessinger, J., E. L. Elson, W. W. Webb, I. Yahara, U. Rutishauser, and G. M. Edelman. 1977. Receptor diffusion on cell surfaces modulated by locally bound concanavalin A. *Proc. Natl. Acad. Sci. U. S. A.* 74:1110–1114.
11. Smith, B. A., W. R. Clark, and H. M. McConnell. 1979. Anisotropic molecular motion on cell surfaces. *Proc. Natl. Acad. Sci. U. S. A.* 76:5641–5644.
12. von Tscharner, V., and H. M. McConnell. 1981. Physical properties of lipid monolayers on alkylated planar glass surfaces. *Biophys. J.* 36:421–427.
13. Weis, R. M., K. Balakrishnan, B. A. Smith, and H. M. McConnell. 1982. Stimulation of fluorescence in a small contact region between rat basophil leukemia cells and planar lipid membrane targets by coherent evanescent radiation. J. Biol. Chem. In press.