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Lateral distribution and diffusion of the C3b receptor of complement, HLA antigens, and lipid probes in peripheral blood leukocytes

(photobleaching/patching/fluorescence/monoclonal antibody)

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ABSTRACT **Fluorescence microscopy and fluorescence redistribution after pattern photobleaching have been used to measure the distribution and motion of a number of fluorescent molecules bound to the plasma membranes of human leukocytes. The fluorescent molecules include fluorescein-labeled $F(ab')_2$ and Fab' fragments of an anti-C3b receptor antibody, fluorescein-labeled IgG and Fab fragments of a monoclonal anti-HLA antibody, and the two lipid probes 3,3'-dioctadecylindocyanine and *N*-4-nitrobenzo-2-oxa-1,3-diazole L-α-dimyristoyl phosphatidylethanolamine. From these studies we have concluded that the C3b receptors on human polymorphonuclear leukocytes and monocytes are predominantly present in discrete clusters. No diffusive motion of these clusters could be detected. In contrast, HLA antigens have a dispersed distribution and have diffusion coefficients between 10^{-9} and 10^{-10} cm²/sec in these peripheral blood leukocytes. Cell-membrane-bound monoclonal IgG anti-HLA undergoes a rapid patching in all these leukocytes except lymphocytes. In about 50% of the lymphocytes, fluoresceinated IgG diffuses slowly, if at all, whereas in the other 50%, fluoresceinated IgG diffuses with a diffusion coefficient of 6.9×10^{-10} cm²/sec. The two above-mentioned fluorescent lipid probes have the usual rapid lipid diffusion coefficient ($\approx 10^{-8}$ cm²/sec) in leukocytes.**

There have been numerous reports of lateral diffusive motions of membrane lipids and proteins in both intact cells and model membranes. Several studies have sought to correlate this mobility with physiological function; for example, the activation of the first component of complement (1), complement depletion (2), macrophage function (3, 4), polymorphonuclear (PMN) leukocyte stimulation (4), mast cell activation (5), and hormone responses (6, 7) have been examined. In the present work we have systematically studied the surface distribution and lateral diffusion of C3b receptors, HLA antigens, and lipid probes in the plasma membranes of several human peripheral blood leukocytes. Most C3b receptors in monocytes and PMN leukocytes are clustered, which may be related to their physiological function. We have also found that HLA antigens are dispersed and diffuse moderately rapidly and that in some leukocytes these antigens are rapidly patched by monoclonal anti-HLA antibody. This latter response to crosslinking may also have physiological significance.

MATERIALS AND METHODS

Preparation of Anti-C3b Receptor Antibody. C3b receptor was solubilized from membranes of human erythrocytes by use of Nonidet P-40 (Gallard–Schlessinger, Carle Place, NY) and was purified by sequential cation-exchange chromatography, affinity chromatography on Sepharose C-3, and gel filtration and affinity chromatography on Sepharose lentil lectin (Sigma). The purified glycoprotein was homogeneous when analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis, presenting a single stained band with an apparent M_r of 205,000 (8). A rabbit was immunized with 75 μg of purified C3b receptor emulsified in Freund's complete adjuvant and was boosted 5 weeks later by intramuscular injection of an additional 50 μg in Freund's incomplete adjuvant. IgG was purified by ammonium sulfate precipitation of the antiserum followed by chromatography on DE52 cellulose. The IgG was digested (9) with pepsin (Worthington) and the $F(ab')_2$ fragments were isolated by gel filtration on Sephadex G-100 (Pharmacia) and by adsorption with Sepharose-protein A (Sigma). Fab' was prepared by mild reduction and alkylation of $F(ab')_2$ (9) followed by gel filtration on Sephadex G-100. Serum obtained from the rabbit prior to immunization was processed in an identical manner to prepare preimmunization IgG, $F(ab')_2$, and Fab'. Monospecificity of the rabbit antibody for C3b receptors was established. It inhibited in a dose-dependent manner the capacities of human erythrocytes, PMN leukocytes, B lymphocytes, and monocytes to form rosettes with sheep erythrocytes bearing C3b and had no effect on C3bi and C3d receptor-dependent rosette reactions by B lymphocytes and monocytes. There was specific binding of ^{125}I-labeled $F(ab')_2$ anti-C3b receptor by these four cell types expressing C3b receptors, but not by T lymphocytes or platelets. The antibody immunoprecipitated only a membrane protein of 205,000 daltons from each of the four cell types that had been surface radioiodinated (10).

Anti-HLA Antibody. Monoclonal anti-HLA-A,B,C (W6/32) was purchased from Accurate Chemical and Scientific (San Diego, CA). This antibody is specific for the 43,000-dalton glycoprotein of chain HLA-A,B,C (11). The antibody was purified by affinity chromatography on a protein A-Sepharose column (12). Fab fragments were prepared by papain digestion in phosphate-buffered saline (pH 7) containing 10 mM EDTA and 20 mM cysteine as described (13). Intact IgG and Fc fragments were removed by protein A chromatography; the void fraction was collected and concentrated by vacuum dialysis. The anti-HLA and anti-C3b receptor antibodies and fragments were shown to be pure by $NaDodSO_4$/polyacrylamide gel electrophoresis.

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Abbreviations: PMN, polymorphonuclear; diI, 3,3'-dioctadecylindocyanine; NBD-PtdEtn, *N*-4-nitrobenzo-2-oxa-1,3-diazole L-α-dimyristoyl phosphatidylethanolamine; FITC, fluorescein isothiocyanate.

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Fluorochrome Conjugation. Antibodies and their fragments were conjugated to fluorescein isothiocyanate (FITC) as described (14). The material was purified by chromatography on Sepharose G-25.

Preparation of Cells. Whole blood was drawn from healthy donors into heparinized vacutainers. Leukocytes were purified as described (15). Briefly, erythrocytes were sedimented for 40 min at room temperature after the addition of 6% (wt/vol) dextran (high molecular weight fraction; Baker) in Hanks' balanced salt solution (GIBCO). The upper layer was washed twice and resuspended in Hanks' solution. This cell suspension was then separated by step-density gradient centrifugation on lymphocyte separation medium (Bionetics, Kensington, MD) at $\approx 400 \times g$ for 40 min at room temperature. The interfacial layer, which contained mononuclear cells, and the pellet, which was made up of erythrocytes and PMN leukocytes, were washed twice and resuspended in Hanks' solution. The cells of the interfacial layer were sometimes separated into adherent (principally monocytes) and nonadherent (principally lymphocytes) fractions by incubation at 37°C in an incubator for 1 hr on petri dishes. When adherent mononuclear cells were used, glass slides or coverslips were placed in the dishes to facilitate microscopic examination. PMN leukocytes were used either directly after the centrifugation above or after enrichment following distilled water or NH_4Cl lysis of erythrocytes. Cell viability was routinely assessed by trypan blue exclusion; in all cases, this was ≥95%.

Fluorescent Labeling of Cells. The lipid analogue 3,3'-dioctadecylindocyanine (diI) was a generous gift of Alan Waggoner. The lipid *N*-4-nitrobenzo-2-oxa-1,3-diazole L-α-dimyristoyl phosphatidylethanolamine (NBD-PtdEtn) was obtained from Avanti Biochemicals. In experiments using lipid probes, 1 ml of cells ($\approx 10^5$ cells per ml) in phosphate-buffered saline at room temperature was rapidly added to 10 μl of a 300 μM lipid solution in ethanol followed by incubation for 5 min at room temperature. The cells were then thoroughly washed and examined as described below. Fluorescence was excited with either the 4880-Å (fluorescein, NBD-PtdEtn) or the 5146-Å (rhodamine, diI) line of the laser. In typical experiments, 5–10 μl of fluorescent antibody was added to 25 μl of cells (10^7 cells per ml) at 4°C and incubated for 10 min on ice unless otherwise noted. The cells were then washed rapidly by three 1-min centrifugations at $500 \times g$ and resuspended in 100 μl of phosphate-buffered saline. Ten microliters of the labeled cell suspension was deposited into a ring of vacuum grease on a microscope slide. The coverslip was pressed on and the slide was examined. When using the water immersion objective, 10 μl of the cell suspension was deposited on the microscope slide, phosphate-buffered saline was added to give a convenient volume, and the objective was immersed into the buffer.

Diffusion Measurements. Measurements were made by the fluorescence recovery after pattern photobleaching technique as described (16) except that some further refinements of the apparatus have been made. A brief description of the present apparatus follows.

The beam from an argon-ion laser (Spectra Physics no. 164-05) is divided into two independent parts by using two 10% beam splitters and two mirrors to split and recombine the beam. The weaker (observation) beam constitutes approximately 1% of the initial laser power and the stronger (bleach) beam approximately 80%. Typical laser power was 400 mW. Both beams are separately controlled by electronic shutters, and attenuating filters may be placed in the observation beam to attain the desired intensity without diminishing the intensity of the bleach beam. The recombined beam is expanded with a pair of lenses to 1–2 cm in diameter and reflected into the rear of the microscope (Zeiss Photomicroscope III, equipped with epifluorescence) from a mirror mounted on the microscope. It then passes through a Ronchi ruling which is imaged onto the fluorescent sample to give a striped pattern. Measurements reported here were made with either a 40× (numerical aperture = 0.65, air) planachromat objective, or a 63× (numerical aperture = 1.2, water) Plan-Neofluar objective, focusing 8.7-μm and 5.5-μm period striped patterns onto the samples.

The fluorescence was measured with a cooled photomultiplier tube (RCA no. C31034-05) in either of two modes, analog or photon counting. The signal was displayed on a storage oscilloscope and also sent to a computer (Digital PDP8/E) for storage and analysis. For measurements of a total time duration greater than 7 sec, the observation beam was chopped to minimize bleaching of the sample. Typically, 100 data points of 25 msec in length were taken. Under these conditions no measurable bleaching by the observation beam was detected.

Data were analyzed by computer least-squares fit to a single exponential to give the time constants for recovery. Diffusion constants were calculated by using the relationship

$$D = 1/a^2\tau, \qquad [1]$$

in which a is the spatial frequency of the pattern and τ is the time constant for exponential recovery (16, 17).

RESULTS

C3b Receptor. In Fig. 1 is shown a representative fluorescence photomicrograph of FITC–$(Fab')_2$–anti-C3b receptor antibody bound to the PMN leukocyte cell surface. The cells were labeled and washed at 0–4°C and observed at room temperature. A similar distribution of fluorescence was obtained with Fab' fragments. The fluorescence was localized in discrete patches on the cell surface. Similar results were obtained with monocytes. $NaDodSO_4$/polyacrylamide gel electrophoresis of the Fab' fragment showed it to be free of detectable IgG. Therefore, the distribution observed is not due to crosslinking of the receptor molecules by bivalent antibody, nor are interactions with the leukocyte Fc receptors involved. As indicated in Table 1, no redistribution of fluorescence after photobleaching was observed, giving an approximate upper limit of $D = 10^{-11}$ cm²/sec for the lateral diffusive motion of the C3b receptors. A rhodamine-conjugated goat $F(ab')_2$ fragment

FIG. 1. Fluorescence photomicrograph of FITC-conjugated rabbit $F(ab')_2$ anti-C3b receptor PMN leukocytes. A similar distribution of fluorescence is obtained with the Fab' fragments. This photograph was taken at ASA 6300 (2475 recording film, push processed) with an intense laser illumination and a relatively long exposure time (≈10 sec). Under these conditions the fluorescent spots appear to be somewhat larger than their actual size. (Bar = 10 μm.)

Table 1. Diffusion of fluorescent probes on leukocyte cell surfaces

Sample	D*	% of cells showing measurable diffusive recovery
FITC-Fab α-C3b receptor on PMN and monocytes	$<10^{-11}$	0
FITC-Fab α-HLA on PMN	$1.5 \pm 0.5 \times 10^{-10}$	100
FITC-IgG α-HLA on lymphocytes†	$6.9 \pm 4.8 \times 10^{-10}$	≈50
DiI in PMN	$1.7 \pm 0.8 \times 10^{-8}$	100
NBD-PtdEtn in PMN	$2.3 \pm 0.5 \times 10^{-8}$	100
DiI in lymphocytes	$0.92 \pm 0.44 \times 10^{-8}$	100

* Reported values of D represent an average of five measurements.
† Measurements showing no recovery on this time scale were not included in average; as discussed in text, only ≈50% of the lymphocyte population shows diffusion of HLA at a measurable rate ($D > 10^{-11}$ cm^2/sec).

against rabbit F(ab′)$_2$ was used to label F(ab′)$_2$ anti-C3b receptor bound to PMN leukocytes at 4°C. The anti-C3b receptor antibody fragment was accessible to the rhodamine-conjugated antibody fragment, and the resulting rhodamine fluorescence showed that the C3b receptor clusters were not internalized. In some of these experiments, the F(ab′)$_2$ anti-C3b receptor was conjugated with FITC. With the use of appropriate fluorescence emission filters to view selectively fluorescein or rhodamine, it was found that these fluorescent molecules had the same spatial distribution. FITC-conjugated preimmunization F(ab′)$_2$ and Fab′ gave no significant amount of labeling. Therefore, the C3b receptors were not internalized in the time course of our experiments (15–30 min). This result is consistent with results of experiments at 4°C with ^{125}I-labeled F(ab′)$_2$ anti-C3b receptor antibody bound to the C3b receptor, which has been found to be exposed on the external surface of PMN leukocytes as judged by susceptibility to proteolytic cleavage.

HLA Antigens. Fluorescence microscopic observations of FITC–Fab–anti-HLA-A,B,C antibody on PMN leukocytes showed the fluorescence to be uniformly distributed in all cases. Mouse FITC–Fab′–anti-H-2K^k as well as rabbit FITC–Fab–anti-vesicular stomatitus virus G-protein showed no fluorescent labeling. The bound Fab–anti-HLA fragments diffuse with $D \simeq 5 \times 10^{-10}$ cm^2/sec (Table 1). There is a substantial variability in the intensity of fluorescence observed on cells within any given population. The brightest cells were chosen for diffusion measurements. Similar distributions of fluorescence were observed on monocytes and lymphocytes.

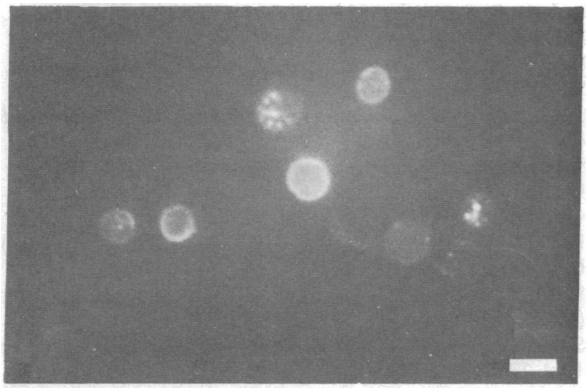

FIG. 2. Fluorescence photomicrograph of FITC-conjugated monoclonal IgG anti-HLA-A,B,C on human lymphocytes and monocytes. The cells with uniform fluorescence have been identified as lymphocytes; the cells with nonuniform fluorescence have been identified as monocytes. This photograph was taken as described in Fig. 1, except that a lower intensity and a shorter exposure time were used. (Bar = 10 μm.)

Fluorescent IgG monoclonal antibody against HLA antigens patched rapidly (within 5 min at 4°C) and extensively on monocytes and PMN leukocytes (but not on lymphocytes). Some of these "patches" were not accessible to the rhodamine-labeled F(ab′)$_2$ fragment of rabbit anti-mouse IgG antibody and were insensitive to trypsin. Fig. 2 shows a fluorescence photomicrograph of FITC–anti-HLA-A,B,C, on a purified population of monocytes and lymphocytes. There are clearly two populations of cells, those in which the fluorescence is highly patched and nonuniform and those in which the fluorescence remains uniform and diffuse. Examination of the cells under transmitted light clearly identified the patched cells as monocytes and the uniform cells as the smaller, rounder lymphocytes. This was substantiated by examining the adherent and nonadherent mononuclear cell fractions. When PMN leukocytes were labeled with this antibody, they also gave a highly patched and nonuniform distribution of fluorescence. Addition of 1 mM *N*-ethylmaleimide substantially reduced the degree of IgG anti-HLA-mediated patching of HLA on monocytes.

The lateral motion of the anti-HLA antibody on lymphocytes was measured by the fluorescence recovery after pattern photobleaching technique. [Earlier studies of H-2 and HLA motions on mouse–human heterokaryons were made by Edidin and Wei (18).] There was significant variation in the rate of motion of the HLA antigens on these cells. Approximately 50% of the lymphocytes showed no recovery on the time scale of our measurements ($D < 10^{-11}$ cm^2/sec), whereas the rest did show recovery, corresponding to $D = 6.9 \times 10^{-10}$ cm^2/sec (Table 1). This heterogeneity in the lymphocyte population was observed in two ways: (*i*) the recovery curves themselves, which sometimes showed no recovery and sometimes showed substantial recovery, and (*ii*) bleaching stripes on cells and observing the disappearance of the stripes visually. In both cases it was clear that there were two different cell populations which exhibited different rates of motion of the monoclonal anti-HLA antibody. Thirty-two cells were pattern bleached; 15 of them showed redistribution of fluorescence and 17 did not on the time scale of the observation (≈200 sec) (see Table 1).

Lipid Probes. The lipid probe dil was found to be uniformly distributed on the surfaces of lymphocytes and PMN leukocytes. The diffusion coefficient of dil was similar for each of these cell types; the values were $1.7 \pm 0.8 \times 10^{-8}$ cm^2/sec and $0.92 \pm 0.44 \times 10^{-8}$ cm^2/sec for PMN leukocytes and lymphocytes, respectively.

We have also assessed the lateral diffusion of the fluorescent phospholipid NBD-PtdEtn in PMN leukocytes. The diffusion coefficient is $2.3 \pm 0.5 \times 10^{-8}$ cm^2/sec, which is not significantly different from that of dil.

DISCUSSION

One principal conclusion of this study is that C3b receptors are predominantly clustered in the membranes of monocytes and PMN leukocytes as measured by Fab′ fluorescence. In contrast, HLA antigens and the lipid probes dil and NBD-PtdEtn are randomly distributed and diffuse laterally on the cell surface. Therefore, the C3b receptors represent a specialization of the cell surface.

Monocytes, macrophages, B lymphocytes, human erythrocytes, and PMN leukocytes possess receptors for C3b, a cleavage fragment of the third component of complement (for a review

see ref. 19). This receptor mediates attachment to the cells of soluble complexes and particles bearing C3b and may facilitate phagocytosis by macrophages and PMN leukocytes. The C3b receptor was isolated (8) and shown to be a glycoprotein with an apparent M_r of 205,000. Previous studies related to the distribution of C3b receptors have used indirect means such as treatment with native C3 followed by anti-C3 sera (20) and rosette formation assays (21). In the present study using monospecific anti-receptor antibody, we have demonstrated that most C3b receptors are present on monocytes and PMN leukocytes in clusters. Clusters of the label were found under all of the conditions used: (*i*) $F(ab')_2$ fragments to avoid interference with Fc receptors, (*ii*) Fab' fragments to avoid crosslinking, and (*iii*) incubation on ice and observation at 6°C to avoid membrane activity (in addition to 24°C and 37°C). Moreover, the cells were further checked by double-labeling experiments to show that the anti-C3b receptor antibody fragments were accessible to a second step $F(ab')_2$ antibody. We conclude that the C3b receptors are present on the cell surface in clusters as measured by Fab' fluorescence.

The clusters of C3b receptors may be structurally somewhat analogous to membrane clusters of the acetylcholine receptor, gap junction proteins, and bacterial rhodopsin. The physiological significance of this clustering may be related to the tendency of C3b to be deposited in clusters on cells such as sheep erythrocytes (22) that have activated complement. If this were a general feature of C3b on target membranes, it would suggest a structure–function correlation. A study of C3b binding and lateral diffusion in hapten-containing model membrane targets might resolve this issue.

Our observed lateral diffusion coefficient of diI on lymphocytes is similar to that previously reported (23). In addition, we have measured the lateral diffusion of diI and NBD-PtdEtn on PMN leukocytes (Table 1). These results show the usual large lipid diffusion coefficients and, at least for diI, uniform labeling of the cell membranes.

Table 1 gives the lateral diffusion coefficient of FITC–Fab–anti-HLA on PMN leukocytes. This diffusion coefficient is similar to those reported for many other intrinsic membrane proteins (24). The distribution of this Fab fragment was uniform on the PMN leukocyte cell surface.

The lateral diffusion coefficient (6.9×10^{-10} cm^2/sec) of monoclonal FITC–IgG anti-HLA on approximately 50% of the lymphocytes is also typical. The other 50% of the lymphocytes were labeled uniformly by monoclonal FITC–IgG anti-HLA but showed no detectable redistribution of fluorescence following pattern photobleaching. The above results were not unanticipated in view of previous studies of intrinsic membrane proteins (24) except for the observation that ≈50% of the lymphocytes were uniformly labeled by monoclonal FITC–IgG anti-HLA and yet showed substantially less redistribution of fluorescence after photobleaching. In a number of experiments (not shown) we attempted to determine if the variation in recovery is correlated with the presence or absence of Fc receptors, using rosetting methods to distinguish between Fc^+ and Fc^- cells (25). No correlation was found.

An additional unexpected observation made in the present work is that monoclonal anti-HLA antibody gives rise to a very rapid patching of the HLA antigen in all the cell types studied except lymphocytes. Because the 43,000-dalton HLA molecule (along with the 12,000-dalton β_2 microglobulin) (11) is expected to have only a single epitopic site for the binding of monoclonal antibody, the observed patching of HLA by this antibody might arise because (*i*) the HLA molecules are dimeric or oligomeric due to disulfide bridges (26–29) or binding to other proteins (or both), or (*ii*) crosslinked pairs of HLA molecules spontaneously patch, or (*iii*) crosslinked pairs of HLA molecules somehow trigger a cellular response leading to the patching of HLA antigens. Because a number of other cell surface immune response-related antigens are not readily patched and capped even with multispecific IgG antisera (30), it is possible that the very rapid patching of HLA by monoclonal anti-HLA is related to some specific biological function. We have not eliminated the possibility of an interaction of the Fc stem with Fc receptors in the PMN leukocytes and monocytes. A related problem of this type has been considered by Unanue (31).

In the present paper we have described cell surface proteins that are (*i*) intrinsically patched and immobile (C3b receptors), (*ii*) uniformly distributed and relatively immobile (HLA on some lymphocytes), (*iii*) uniformly distributed and mobile (HLA on other lymphocytes), and (*iv*) rapidly patched by monoclonal antibodies (HLA on PMN leukocytes and monocytes). These results suggest the possibility of a functional diversity related to the distribution and mobility of these intrinsic membrane proteins.

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