Aberrant Capping of Membrane Proteins on Neutrophils From Patients With Leukocyte Adhesion Deficiency

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Several functional defects have been found in neutrophils from leukocyte adhesion deficiency (LAD) patients who fail to express the CD11/CD18 leukoadhesins: Mo1, LFA-1, and p150,95. To better understand the functional defects of LAD neutrophils, we have performed capping experiments. Purified normal or LAD neutrophils were labeled with fluorochrome-conjugated concanavalin A (Con A) or F(ab)2 fragments of anti-FcγRII (CD18), anti-Mo5, and anti-CD14 antibodies. F(ab)2-labeled cells were capped using a second-step F(ab)2 fragment of an antitrust mouse Fab antiserum. Cells were capped for 30 minutes at 37°C, then observed by fluorescence microscopy. LAD neutrophils were found to be deficient in capping, but not clustering.

LEUKOCYTE ADHESION deficiency (LAD) is a rare inherited disorder of leukocyte functions that is characterized by recurrent life-threatening infections. Al although defects in both lymphocytes and phagocytes have been reported, the latter contribute to the clinically significant bacterial infections seen in patients. Neutrophils from LAD patients exhibit defective aggregation, chemotaxis, phagocytosis, antibody-dependent cellular cytoxicity, and binding to surfaces including iC3b-coated surfaces, glass surfaces, and endothelial cell surfaces. These functional defects are associated with a partial or complete absence of the membrane-bound CD11/CD18 glycoproteins that are members of the integrin supergene family. The CD11/CD18 leukoadhesins are a family of three heterodimeric transmembrane proteins known as Mo1 (complement receptor type 3 [CR3]), lymphocyte function-associated antigen 1, and p150,95. Both deletions and point mutations within the CD18 gene have been linked to faulty plasma-membrane expression of these molecules, thus causing disease. Therefore, a broad spectrum of cellular defects result from the defective expression of CD11/CD18 glycoproteins.

To better understand the defective membrane functions of LAD neutrophils, we have performed capping studies of both normal and LAD neutrophils. The capping properties of all of the reagents tested to date. The percent of cells exhibiting capping of Con A, FcγRII, urokinase receptor, CD14, and Mo5 were 52%, 67%, 70%, 25%, and 64% for normal neutrophils but were only 10%, 5%, 2%, 3%, and 1%, respectively, for LAD neutrophils. Capping of this panel of membrane components in LAD or normal neutrophils was not augmented by the addition of either 10−8 mol/L colchicine or 10−7 mol/L FMLP. Because capping requires membrane-to-cytosol communication and an intact microfilament linkage, we suggest that leukoadhesins may play a broad role in promoting the redistribution of membrane components including adherence-related receptors such as FcγRII and the urokinase receptor.

MATERIALS AND METHODS

Materials. FMLP, fluorescein-conjugated concanavalin A (Con A), and colchicine were obtained from Sigma Chemical Co. (St Louis, MO). Individually packaged aliquotes of fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) were obtained from Molecular Probes (Eugene, OR).

Monoclonal antibody (MoAb). F(ab)2 fragments of a MoAb (3G8) directed against FcγRII (CD16) were obtained from Medarex Inc (West Lebanon, NH). Mouse MoAbs to a nonpolymorphic class II determinant (clone 9-49ga), Mo5, and CD14 (clone 2B10) and their fragments were prepared as previously described. Goat F(ab)2 fragments of antimouse F(ab)2 fragments and anti-ι2-microglobulin were purchased from Cappel-Organon Teknika Corp (Malvern, PA).

Preparation of FITC- and TRITC-conjugated antibodies. The TRITC- and FITC-conjugated antibodies were prepared as described, with minor modifications. Briefly, antibody fragments were dialyzed against phosphate-buffered saline (PBS) at pH 7.4 for 2 hours, then dialyzed against 0.15 mol/L carbonate-bicarbonate buffer, pH 9.3, for 4 hours at 4°C. The antibodies were incubated with fluorochrome at a fluorochrome/protein (F/P) ratio of 40 μg TRITC and 30 μg FITC/mg antibodies at room temperature for 4 hours. The fluorescent conjugates were separated from unreacted fluorochromes by Sephadex G-25 (Sigma) column chromatography. Purified conjugates were dialyzed against PBS at pH 7.4 overnight at 4°C. All conjugates retained tissue specificity.

Preparation of neutrophils. Peripheral blood was obtained from healthy adults using heparinized tubes. Blood was also drawn from two patients previously characterized as having LAD. CD18 expression was approximately 1% of normals as determined by flow cytometry. Neutrophils were isolated by Ficoll-Hypaque (Sigma) step-density gradient centrifugation. The remaining red blood cells were depleted by hypotonic lysis. The cell preparation was approximately 95% polymorphonuclear leukocytes. The purified neutrophils were typically 95% viable, as assayed by trypan blue exclusion.

Capping. Cells were capped as previously described. Briefly, purified normal or LAD neutrophils (7 × 106 in 100 mL of Hanks' Balanced Salt Solution [HBSS]) were stained at 4°C with TRITC-conjugated F(ab)2 fragments of anti-FcγRII antibodies or FITC-conjugated F(ab)2 fragments of anti-Mo5 antibodies (March 15, 1994). pp 1650-1655
labeled F(ab')2 fragments of anti-uPAR, anti-CD14, anti-Mo5, or anti-β2-microglobulin antibodies. After staining, the cells were washed with cold HBSS by centrifugation. No qualitative differences in the level of immunofluorescent staining were observed for these labels on LAD and normal neutrophils. In several experiments, samples of cells were treated with 10−3 mol/L colchicine for 30 minutes at 4°C. Cells were then incubated with goat F(ab')2 fragments of goat-antimurine F(ab')2 antiserum for 30 minutes at 4°C. The cells were then washed twice with HBSS by centrifugation and incubated at 37°C for 30 minutes. In other experiments, cells were treated with 10−3 mol/L FMLP during incubation with the second-step reagent or during incubation with the second-step reagent and capping at 37°C for 30 minutes. After these procedures the cells were treated with HBSS containing 0.1% sodium azide to inhibit further capping before microscopic observations.

Microscopy. Cells were examined using an automated axiovert inverted fluorescence microscope (Carl Zeiss, Batavia, IL) with mercury illumination interfaced to a Percepets Biovision System (Knoxville, TN) as previously described.23,24 Fluorescence micrographs were collected by a Hamamatsu intensified charge-coupled device camera (Model C2400-97; Carl Zeiss). Narrow bandpass discriminating filter sets were used to allow excitation at 485/22 nm and emission at 530/30 nm for FITC and excitation of 540/20 nm and emission of 590/30 nm for TRITC (Omega Optical, Brattleboro, VT). Differential interference contrast photomicrographs were obtained using Zeiss polarizers and a charge-coupled device camera (Model 72; Dage-MTI, Michigan City, IN). The background-subtracted digitized images were averaged then stored on hard disk or streaming tape. Processed images were recorded using a Polaroid (Boston, MA) freeze-frame video recorder. Approximately 200 to 700 randomly selected cells were counted for each experimental trial. Capped cells were defined as those cells that expressed the label as a large cluster in a polar fashion. The capping percentage is defined as the number of cells showing capping, divided by the total cells counted, multiplied by 100. To determine if the cell-associated fluorescence was associated with the exterior cell surface, crystal-violet quenching was performed as previously described.25

RESULTS

Parallel capping studies were performed on normal and LAD neutrophils, as described in Materials and Methods. F(ab')2 fragments of antibody molecules were used in this study to promote cross-linkage of membrane proteins without potential interference of Fc-domain Fc-receptor interactions. The capping of transmembrane proteins, glycosphospholipid-linked proteins, and lectin binding sites were examined in this study. To augment capping of membrane proteins, the reagent colchicine was also used in these studies.26 The capping properties of glycosphospholipid-linked proteins uPAR, FcyRIII, and CD14 on neutrophils was examined. Normal neutrophils readily capped uPAR in the absence (70% ± 10%) and presence (80% ± 10%) of colchicine at 10−5 mol/L, as described above (Table 1). In contrast, LAD neutrophils displayed little capping of uPAR (2% ± 2% and 3% ± 2% capping without and with colchicine, respectively). Figure 1 shows representative micrographs of LAD (panels A and B) and normal (panels C and D) neutrophils after exposure to uPAR capping conditions. Although considerable patching was induced by the multiple crosslinking stimuli, capping was not observed on LAD neutrophils (Fig 1, A and B; Table 1). Similar results were obtained for FcyRIII (Fig 1, E to H; Table 1). Normal neutrophils readily capped FcyRIII with or without colchicine (78% ± 8% and 67% ± 10% capping, respectively) whereas LAD cells did not (7% ± 3% and 5% ± 2% capping, respectively). The membrane capping properties of CD14 were also examined on these cells (Fig 1, I to L; Table 1). These results show that the glycosphingolipid-linked proteins uPAR, FcyRIII, and CD14 are defective in plasma-membrane capping on LAD, but not normal, neutrophils.

The capping properties of two transmembrane proteins were also examined (Table 1). The capping percentages were determined for both normal and LAD neutrophils in the presence and absence of colchicine using anti-Mo5 and anti-β2-microglobulin reagents. Mo5 is a 50-kD glycoprotein that is insensitive to phosphatidylinositol (PI)-specific phospholipase C (data not shown). As Table 1 shows, LAD neutrophils displayed little or no capping of these reagents in the presence or absence of colchicine. However, moderate to high levels of capping were found using normal neutrophils. Representative micrographs of capping experiments are shown in Fig 1, M to T. Again, patching is observed for LAD cells (Fig 1, M, N, Q, and R). In contrast, normal neutrophils capped these reagents under identical conditions (Fig 1, O, P, S, and T). The greatly diminished capping ability of LAD neutrophils was significant in all comparable studies. This indicates that the deficiency in LAD cell capping is not restricted to a single membrane protein or type of membrane protein.

The ability of Con A to cap on normal and LAD neutrophils was also examined (Fig 1, U to X; Table 1). In comparison with the other reagents tested on LAD neutrophils, Con A exhibited the highest capping level (10% ± 2% and 14% ± 4% capping in the absence and presence of colchicine, respectively). However, this level of capping remained significantly lower than that of normal neutrophils (52% ± 12% and 64% ± 10% capping in the absence and presence of colchicine, respectively). Although the morphology of the caps on normal neutrophils are clearly associated with plasma-membrane fluorescence, the punctate fluorescence patterns of LAD neutrophils could be associated with internalization of the labels. Hence, to distinguish plasma-membrane patching from adsorptive pinocytic uptake of the fluorescent labels, we performed similar experiments that incorporated the use of crystal violet to quench extracellular fluorescence.25 These experiments showed that the labels were associated with the cell surface, not cytoplasmic structures (data not shown). Therefore, the inability of LAD neutrophils to cap these labels was not caused by enhanced interiorization of the label.

Because CR3 has been reported to participate in transmembrane calcium signaling,27 it seemed possible that the capping deficiency of LAD neutrophils was caused by a missing calcium signal. To test this possibility, FMLP was included in capping experiments to provide a calcium signal.28 FMLP at 10−7 mol/L in HBSS was added to cells during treatment with the second-step F(ab')2 fragment or during both second-step treatment and capping protocols. Identical results were obtained in both cases. The addition of FMLP did not restore the ability of LAD cells to cap any
member of this panel of reagents (Table 1). Furthermore, 10^{-7} mol/L FMLP did not augment the observed level of capping in normal neutrophils (Table 1). The reduction in Con A capping mediated by FMLP in normal neutrophils in comparison with colchicine-treated normal neutrophils is consistent with a previous report. Therefore, the lack of calcium signaling may not be responsible for the observed defect in capping.

**DISCUSSION**

A principal conclusion of this study is that LAD neutrophils possess a profound deficiency in their ability to cap a broad spectrum of membrane components, although they retain the ability to patch membrane components. This capping defect was evident for transmembrane proteins, glycoprophospholipid-linked proteins, and lectin binding sites. The broad nature of defective capping is consonant with the broad influence of this disease on membrane functions such as phagocytosis, chemotaxis, adherence, spreading, and cytotoxicity.

Recent studies from this laboratory indicate that FcγRIII and CR3, which is missing in LAD neutrophils, collaborate with one another to generate cocapping on neutrophil membranes, cytosolic calcium transients, superoxide production, and transmembrane cytoskeletal links. Because transmembrane links and the cytoskeleton are necessary to cap membrane proteins, and because we have postulated that CR3 provides a transmembrane link for the glycophospholipid-linked FcγRIII molecules, we tested the hypothesis that LAD neutrophils would express a deficiency in FcγRIII capping. Our results show that FcγRIII capping is defective in LAD but not normal neutrophils, thus providing further evidence that CR3 plays an important role in regulating the behavior of FcγRIII. Importantly, we have also found that a panel of reagents, including antibody fragments and the lectin Con A, exhibit defective capping on LAD neutrophils. Given the broad range of aberrant membrane functions in LAD neutrophils, this is not completely unexpected.

The molecular interactions leading to the capping defect are not certain. However, it seems likely that CR3 does not directly interact with all of the membrane proteins we have tested. For example, we have shown that CR3 does not co-cap with Mo5, CD14, and ß₂-microglobulin. Fluorescence quenching experiments ruled out the possibility that the defective capping was caused by rapid interiorization of the label. Furthermore, experiments using the reagent colchicine ruled out the possibility that microtubules, which can restrict the capping of some membrane components, were not responsible for the diminished ability of LAD neutrophils to cap the spectrum of reagents used in this study. This result is consistent with a previous study showing that microtubule assembly in LAD and normal neutrophils are indistinguishable. Because CR3 is known to trigger intracellular calcium signals, we tested that the ability of normal and LAD neutrophils to cap membrane components in the presence of FMLP. Formyl peptide receptors are known to stimulate calcium signaling and participate in actin assembly. We have previously reported that neutrophils from the two LAD patients used in the present study undergo normal calcium transients in response to FMLP. However, FMLP did not restore capping to LAD cells nor did it augment the capping response of normal neutrophils. Hence, calcium signaling does not appear to be a primary factor in the capping defect of LAD neutrophils.
MEMBRANE CAPPING ON LAD NEUTROPHILS

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limiting the ability of LAD cells to cap membrane components. Furthermore, Southwick et al.22 have shown that LAD neutrophils are not defective in FMLP-stimulated actin assembly. Consequently, the deficiency in LAD neutrophil capping is likely not caused by aberrant actin assembly. Therefore, we suggest that the capping deficiency is not caused by restraint by microtubules or by deficiencies in calcium signaling or subsequent actin assembly.

CR3 could play a direct role in capping membrane proteins that it is known to copack with, such as Con A, FcyRIII, and uPAR, thereby explaining the deficiency in capping of these components of LAD neutrophil membranes. However, the potential mechanisms involved in the broader capping deficiency of LAD neutrophils is unknown. These data do suggest that integrins play an important role in capping phenomena. For example, membrane capping may require the formation of focal contact-like structures formed by integrins. A recent study by Wang et al.32 showed that integrins transmit mechanical signals to the cytoskeleton. Hence, the mechanotransduction ability of integrins may play a role in the capping of membrane components brought about by their crosslinkage. Future studies should delineate the broad mechanisms of CR3 in immunologic adhesion and effector functions.

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