Online Data Supplement

Activation of β_1 Integrins on Blood Eosinophils by P-Selectin

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MATERIALS AND METHODS

Antibodies

Monoclonal antibodies to β_1 integrin MAR4 and mAb13, activation-sensitive anti-β_1 N29, activation-sensitive anti-β_2 mAb24, phycoerythrin (PE)-conjugated goat anti-mouse and anti-rat IgG antibodies, FITC-conjugated goat anti-mouse IgG, FITC-conjugated anti-CD14 and anti-CD16, and isotype controls mouse IgG_1 and rat IgG_{2a} were as described (E1, E2). The following additional antibodies were also used. Activation-sensitive anti-β_1 integrin mAb 8E3 (E3) was a gift from Martin Humphries (Wellcome Trust Center for Extracellular Matrix Research, Manchester, UK). Activation-sensitive anti-β_1 9EG7 (E4), anti-P-selectin AC1.2, anti-PSGL-1 KPL-1, and allophycocyanin (APC)-conjugated anti-P-selectin AK-4 were from BD Biosciences (San Jose, CA). Anti-eosinophil major basic protein (MBP) BMK13 and anti-eosinophil peroxidase (EPO) AHE-1 were from Chemicon (Temecula, CA). Anti-α_{IIb} HIP8 was from Exbio (Vestec, Czech Republic). Anti-α_{IIb} VIPL3 was from Invitrogen (Carlsbad, CA). Rhodamine Red X-conjugated goat anti-rat IgG (with minimal reactivity to mouse) and FITC-conjugated donkey anti-mouse IgG (with minimal reactivity to rat) for double immunofluorescence microscopy staining were from Jackson Immunoresearch Laboratories (West Grove, PA). Rhodamine-conjugated avidin was from Miles-Yeda (Israel). Anti-thrombospondin (TSP)-1 HB8432 (E5) was biotinylated as described (E6).

Flow cytometry

Venous blood was drawn into standard lavender-top EDTA tubes (BD Vacutainer Systems, Franklin Lakes, NJ) and directly processed for flow cytometry as described (E2). Eosinophils, neutrophils, and monocytes were gated based on scatter and lack of reactivity with anti-CD14 and anti-CD16 as before (E2). Data are expressed, as before, as specific geometric mean channel fluorescence (gMCF) = 1,024 x log geometric mean fluorescence of the experimental sample – 1,024 x log geometric mean fluorescence of the isotype control (E2, E7). Mid-range one-peak rainbow fluorescent beads (Spherotech, Lake Forest, IL) were run at setup in order to set the sensitivity of the detectors at a standardized fluorescence intensity. Post-data-collection compensation was performed using matrix algebra by FlowJo (TreeStar, Ashland, OR).

For double labeling, blood was incubated with unlabeled primary and phycoerythrin (PE)-conjugated secondary antibodies as usual, followed by resuspension in 100 µl FACS buffer (PBS with 2% BSA and 0.2% NaN_3, pH 7.2) with APC-conjugated anti-P-selectin (20 µl) and incubation for 30 min. After labeling, RBC were lysed and samples were fixed and prepared for data collection as before, and eosinophils were gated based on forward and side scatter (E2). The eosinophil population gated in this manner is 96%-100% pure, judged by immunofluorescence microscopy staining of

sorted and cytospun cells for eosinophil MBP (E2).

Purified eosinophils were incubated, fixed, and analyzed as described (E1), except that PE-conjugated secondary antibody (2 μg/ml) was used. Purified eosinophils or EoL-3 cells non-adherent to VCAM-1 were analyzed live. FITC-conjugated secondary antibody was used, followed by resuspension of cells in FACS buffer containing 1 μg/ml propidium iodide, and immediate data collection. Live cells were gated based on forward scatter and lack of staining with propidium iodide.

Immunofluorescence microscopy

One hundred μl of venous blood, drawn into EDTA tubes, was incubated for RBC lysis with 2 ml of FACS lysing solution (BD Biosciences) for 10 min at room temperature, followed by centrifugation for 10 min at 260 x *g* (1,200 rpm in a Sorvall Technospin R centrifuge, Du Pont, Wilmington, DE). The whole leukocyte pellet was resuspended in 500 μl HBSS containing 2% BSA and centrifuged again at 4°C. Cells were resuspended in 1.5 ml of HBSS-2% BSA and cytospun onto microscope slides for 3 min at 600 rpm. The following steps were performed at room temperature. Cytospun cells were fixed first for 10 min with 1% EM-grade methanol-free paraformaldehyde (EM Sciences, Fort Washington, PA) in 75 mM sodium cacodylate buffer, pH 7.4, containing 0.72% sucrose; and then for 10 min with 3.7% EM-grade methanol-free formaldehyde (Polysciences, Warrington, PA) in PBS; washed by dipping the slide three times in PBS; permeabilized with 0.05% Triton X-100 in PBS for 4 min (for TSP-1, MBP, and EPO staining) or not (for β_1 integrin and cell-surface P-selectin staining); washed; stained; mounted; and sealed under round glass coverslips (12 mm diameter, Fisher Scientific, Pittsburgh, PA) as described (E1). Thirty μl of each fixative or antibody solution was added onto the area of the slide containing the cytospun cells. Primary antibodies, including biotinylated anti-TSP-1, or isotype controls were used at 10 μg/ml. For double labeling of activated or total β_1 integrin and P-selectin, cells were first incubated with a mixture of activation-sensitive rat anti-β_1 9EG7 or rat anti-total β_1 mAb13 and mouse anti-P-selectin and then with a mixture of rhodamine-conjugated anti-rat and FITC-conjugated anti-mouse secondary antibodies. For double labeling experiments that included TSP-1, cells were first incubated with a β_1 or P-selectin primary antibody, second with FITC-conjugated secondary antibody, then permeabilized, third incubated with biotinylated anti-TSP-1, and fourth with rhodamine-avidin. Samples were viewed in a BX60 microscope (Olympus, Melville, NY). An area with at least 500 leukocytes was observed on each coverslip. Representative eosinophils were photographed using a digital camera with the SPOT RT Advanced software (Diagnostic Instruments, Sterling Heights, MI).

Selective adhesion to VCAM-1

Wells in six-well tissue culture treated plates (Corning, Corning, NY) were coated with 1 ml of recombinant soluble extracellular 7-domain-VCAM-1 (E1) in Tris-buffered saline (TBS) for 2 h at 37°C. The wells were washed with TBS, blocked with 0.1% gelatin (for purified eosinophils or whole blood) or 1% BSA (for EoL-3 cells) in TBS for 30 min at 37°C, and washed with TBS. One ml of purified blood eosinophils (4 x 10^6/ml) or EoL-3 cells (2 x 10^6/ml) in RPMI 1640 with 0.2% BSA; or venous blood, drawn from volunteer donors into heparin tubes and preincubated with 0.1 μg/ml P-selectin for 1 h at 37°C; was added to each

well. After incubation for 1 h at 37°C, non-adherent cells were harvested. Non-adherent purified eosinophils or EoL-3 cells were spun down for 10 min at 4°C at 1,200 (eosinophils) or 1,500 (EoL-3 cells) rpm, resuspended in 400 µl FACS buffer, and processed for flow cytometry. Non-adherent blood cells were processed directly for flow cytometry as described above without an intermediate centrifugation step.

Adhesion under static conditions

Wells in polystyrene non-tissue culture treated 96-well culture plates (BD Labware, Franklin Lakes, NJ) were coated with 100 µl of 1 µg/ml recombinant soluble extracellular 7-domain- or 6-domain-VCAM-1 (E1, E8) in TBS, pH 8.0, for 2 h at 37°C, decanted, blocked with 0.1% gelatin in TBS for 30 min at 37°C, and decanted. One hundred µl of venous blood, drawn from volunteer donors into heparin tubes, preincubated for 1 h at 37°C with or without different concentrations of P-selectin was added to each well and incubated for 1 h at 37°C, after which the wells were washed six times with TBS. Adherence of eosinophils was quantified by an EPO assay as described, with a chromogenic substrate and measurement of the absorbance of the colored product spectrophotometrically at 490 nm (E7, E8).

References

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Figure Legends

Figure E1. Flow cytometric analysis of expression of anti-PSGL-1 or epitopes for activation-sensitive β_1 integrin mAbs N29, 8E3, or 9EG7 versus anti-P-selectin on eosinophils in whole blood from a subject with non-severe allergic asthma. Representative of result of experiments with three subjects. (*A*) mouse IgG_1 isotype (control for *B-D*), (*B*) N29, (*C)* 8E3, (*D*) anti-PSGL-1, (*E*) rat IgG_{2a} isotype (control for *F*), or (*F*) 9EG7 versus anti-P-selectin. Note P-selectin-high populations marked with ellipses.

Figure E2. Immunofluorescence microscopic localization of the platelet markers TSP-1 and α_{IIb} integrin, and eosinophil MBP in eosinophils. Representative of results of experiments on cells from seven subjects with non-severe allergic asthma. Eosinophils in cytospun whole leukocytes were identified by positive staining for eosinophil MBP (*B*) or EPO (not shown), and by morphology (C, E). Micrographs of an eosinophil analyzed by staining with biotinylated mAb to TSP-1 and rhodamine-avidin (*A*), mAb to eosinophil MBP and FITC-conjugated anti-mouse secondary antibody (*B*), and by phase contrast (*C*). Micrographs of an eosinophil analyzed by staining with mAb VIPL3 to α_{IIb} and FITC-conjugated anti-mouse secondary antibody (*D*), and by phase contrast (*E*). Note the characteristic eosinophil bilobed nucleus and areas in eosinophils positive for the platelet markers (arrowheads). Scale bar = 5 μm.

Figure E3. Effect of added soluble P-selectin on specific expression of epitope for activation-sensitive anti-β_1 integrin mAb N29 on eosinophils in whole blood. N29 epitope expression after preincubation with (gray bars) or without (white bars) added soluble P-selectin (PS)(100 ng/ml) in normal (N), allergic rhinitic (non-asthmatic)(AR), or allergic asthmatic (AA) donors. $n = 3$ N, 14 AR, and 13 AA donors. Error bars = SEM. $^{**}P \leq 0.01$ versus control without P-selectin.

Figure E4. Effect of added soluble P-selectin or IL-5 on specific expression of epitopes for activation-sensitive anti-β_1 integrin mAb N29 or activation-sensitive anti-β_2 integrin mAb24 on leukocytes in whole blood. N29 (*A,C,E*) or mAb24 (*B,D,F*) epitope expression on eosinophils (*A,B*), neutrophils (*C,D*), or monocytes (*E,F*) after preincubation without (white bars) or with added soluble P-selectin (PS, 100 ng/ml)(light gray bars) or IL-5 (50 ng/ml)(dark gray bars). $n =$ 5 donors whose eosinophils responded to P-selectin. Error bars = SEM. $^{*}P \leq 0.05$ versus control (ctrl).