Online Data Supplement

Platelet activation, P-selectin, and eosinophil β_1 integrin activation in asthma

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METHODS

Assessments

SARP subjects had eight visits over about 14 weeks (Table E1). Spirometry and methacholine challenge conformed to ATS guidelines; predicted lung function values and PC_{20} (provocative concentration of methacholine producing a 20% fall in FEV_1) were computed as described (E1). A PC_{20} value of 50 mg/ml was assigned to subjects with < 20% FEV_1 fall at 25 mg/ml. Total white blood cell, differential, and platelet counts (by the impedance method) were obtained with the XE-2100 Automated Hematology System (Sysmex, Mundelein, IL). Fraction of exhaled nitric oxide (FeNO), sputum induction and analysis were performed as before (E2); samples with > 80% squamous cells were excluded. Total serum immunoglobulin E (IgE) was measured with the Uni-Cap system (Phadia, Uppsala, Sweden). Skin test was performed with twelve allergens (Table 1 legend).

Antibodies

Anti-total β_1 integrin monoclonal antibody (mAb) MAR4, activation-sensitive anti-β_1 mAb N29, anti-P-selectin AC1.2, anti-α_{IIb} integrin (CD41) HIP8, anti-P-selectin glycoprotein ligand-1 (PSGL-1, CD162) KPL-1, phycoerythrin (PE)-conjugated goat anti-mouse immunoglobulin (Ig)G antibody, fluorescein isothiocyanate (FITC)-conjugated anti-CD14 and anti-CD16, and isotype control mouse IgG_1 were as described (E3). FITC-conjugated anti-α_{IIb} integrin mAb VIPL3 was from Invitrogen (Carlsbad, CA). Anti-thrombospondin-1 (TSP-1) mAb HB8432 was as described (E4-6).

Flow cytometry of eosinophils

For analysis of eosinophils, whole, unfractionated blood drawn into vacuum tubes containing CTAD (citrate, theophylline, adenosine, and dipyridamole) anticoagulant solution (BD Vacutainer Systems, Franklin Lakes, NJ) was directly processed for flow cytometry, and data were acquired, analyzed, and are expressed as described (E3). The data presented for each subject are the mean of data from the subject's blood draw for flow cytometry visits. The number of visits analyzed per subject varied due to logistical reasons related to the fact that the blood samples had to be processed immediately. Data were collected from three subjects at three visits, from 15 subjects at two visits, and from the remaining 19 subjects at one visit.

Flow cytometry of platelets

For analysis of P-selectin expression on platelets, flow cytometry was performed according to a slight adaptation of a described method (E7): Five hundred μl of blood drawn into CTAD was centrifuged for 10 min at room temperature at 180 x *g* (1,000 revolutions per minute [rpm] in a Sorvall Technospin R centrifuge, Du Pont, Wilmington, DE). The platelet-rich plasma supernatant was withdrawn, 125 μl was fixed by incubation with 1.25 ml FACS fixative (1% paraformaldehyde, 67.5 mM sodium cacodylate, 113 mM NaCl, pH 7.2) on ice for 1 h. The fixed platelets were centrifuged for 10 minutes at 4°C at 720 x *g* (2,000 rpm in the same centrifuge) and the pellet was resuspended in 500 μl FACS buffer (phosphate-buffered saline [PBS] with 2% bovine serum albumin [BSA] and 0.2% NaN_3, pH 7.2). The following incubations were at 4°C. Platelet suspension (100 μl) was incubated with 0.5 μg anti-P-selectin primary antibody or isotype control in 100 μl of FACS buffer for 30 min. After primary antibody incubation, samples were centrifuged at 2000 rpm for 10 min, and resuspended in 250 μl FACS buffer containing PE-

conjugated anti-mouse IgG (2 μg/ml). After incubation for 30 min, samples were centrifuged again, resuspended in 100 μl FACS buffer with FITC-conjugated anti-α_{IIb} and incubated for 30 min. Samples were centrifuged, resuspended in 250 μl FACS buffer, and data were collected from 10,000 platelets. Platelets were gated inside both characteristic regions in a plot of side scatter versus FITC staining and a plot of side versus forward scatter. As with eosinophils and the other leukocytes (E3), 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. However, in order to optimize the settings with isotype control and due to the different background signals between platelets and leukocytes, the settings for the acquisition of platelet data differed from that for leukocytes.

Enzyme-linked immunosorbent assays (ELISAs)

Plasma was prepared from blood collected in vacuum tubes containing CTAD anticoagulant solution at 10% of the final fill volume. The sample was double-spun to minimize platelet activation and contamination, the first centrifugation for 10 min at room temperature at 100 x *g* (1,000 rpm in an accuSpin Micro 17R centrifuge, Fisher Scientific, Pittsburgh, PA) and the second centrifugation for 10 min at 4°C at 10,000 x *g* (10,000 rpm in the same centrifuge). The final supernatant was stored in aliquots at -80°C until tested. The concentrations of soluble plasma P-selectin and platelet factor 4 (PF4) were determined using sandwich enzyme-linked immunosorbent assays (ELISAs)(R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. For P-selectin analysis, samples were diluted 1:20, 1:50, and/or 1:100, and the value(s) within the range of the standards were averaged. For PF4 analysis, samples were diluted 1:300, and if needed 1:100 and 1:30. The absorbance of the colored

product was measured at 450 nm in a Genios Pro plate reader (Tecan, Männedorf, Switzerland). Each dilution was run in duplicate. The detection limits were 5 and 0.9 ng/ml P-selectin and PF4, respectively.

The concentration of plasma TSP-1 was determined by a competitive ELISA, which was a modified version of a described method (E4). Wells in a polystyrene 96-well plate (Corning, Corning, NY) were coated with 50 μl 1 nM of a recombinant C-terminal segment of TSP-1 (E123CaG1)(E6) in Tris-buffered saline (TBS) with 2 mM Ca^{2+}, pH 7.4, overnight at 4°C. All subsequent steps were performed at room temperature, and wells were washed with TBS containing 2 mM Ca^{2+} and 0.05% Tween-20 (TBST) between all steps. Wells were blocked with 5% BSA in TBST for 1 h. Samples (150 μl) diluted 1:3 (or, if needed, undiluted or diluted up to 1:200) or E123CaG1 used as standard (0.01-30 nM) were mixed with 25 μl biotinylated anti-human TSP-1 mAb HB8432 (E5, E6)(final concentration 10 ng/ml) in TBST with 0.1% BSA, and pre-incubated for 1 h before the competition mixture was added to the blocked wells and incubated for 2 h. The wells were then incubated with 50 μl 2 μg/ml horseradish peroxidase-streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. Finally, 50 μl of SureBlue TMB microwell peroxidase substrate (KPL, Gaithersburg, MD) was added to each well. The wells were monitored until appropriate blue color developed and then 50 μl SureBlue stop solution (KPL) was added. Absorbance was read at 450 nm as above. Each sample was run in duplicate. The detection limit was 1.5 ng/ml TSP-1. In samples from occasional visits (two samples of 72, one subject of 37), the obtained TSP-1 values were very high (> 5,000 ng/ml), likely due to platelet activation during sample acquisition or plasma preparation. These data were excluded from analysis and used to censor PF4 data, *i.e.*, PF4 data from the same visits were also excluded.

Values from ELISAs were not adjusted for the dilution of plasma by the anticoagulant solution present in the CTAD tubes because the correction for this dilution depends on knowing the packed red cell (PRBC) volume of the tube. PRBC volumes of blood collected in 1% EDTA anticoagulant, measured at visit three, were in the normal range of 35-47% of total volume, so ratios of diluted CTAD plasma:undiluted plasma can be assumed to vary minimally from 0.84 to 0.87.

Whole-lung antigen challenge in non-SARP subjects

Sixteen non-SARP subjects with mild physician-diagnosed allergic asthma were screened and underwent a whole-lung inhaled antigen challenge as described (E8). Blood for flow cytometry was drawn into vacuum tubes containing CTAD at 0 h immediately before antigen challenge and at 2, 8, and 48 h after antigen challenge and was processed as above. Blood eosinophil counts in these subjects were obtained with Unopette test 5877 (BD, Rutherford, NJ).

References

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TABLE E1. SARP STUDY DESIGN

Visit	1	2	3	4	5	6	7	8
Consent and medical history	x							
Spirometry	x							
Methacholine challenge		x						
Allergy skin test		x						
Blood draw: IgE, leukocyte and platelet counts			x					
Blood draw: flow cytometry and plasma preparation				x			x	x
FeNO						x		
Sputum induction							x	

Definition of abbreviations: See Table 1.

Inhaled β agonist was withheld for 4 h before Visits 1 and 2.

Visit 4 was at least two weeks after Visit 1, Visit 6 was within four weeks of Visit 4, Visit 7 was six weeks after Visit 4, and Visit 8 was twelve weeks after Visit 4.