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SELECTED ANTIBODIES TO LEUKOCYTE COMMON ANTIGEN (CD45) INHIBIT HUMAN NEUTROPHIL CHEMOTAXIS[1]

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The CD45 Ag family is a group of high m.w. glycoproteins that are expressed on the plasma membranes of all leukocytes. CD45 has protein tyrosine phosphatase activity and appears to regulate signal transduction and lymphocyte activation by specific association with receptor molecules on T and B lymphocytes. However, little is known about CD45 function in neutrophils (PMN). In this study, PMN were incubated with CD45 mAb and tested for their chemotactic responses to four unrelated chemoattractants: FMLP, leukotriene B_4 (LTB_4), recombinant human C5a (C5a), and recombinant human neutrophil-activating protein-1, recently designated IL-8. A panel of CD45 mAb including an IgM mAb, AHN-12.1, and six IgG1 mAb, AHN-12, AHN-12.2, AHN-12.3, AHN-12.4, HLe-1, and KC56(T200), were tested for their effects on PMN chemotaxis. PMN chemotaxis was evaluated with two different membrane assays; one assay quantified the total number of migrating PMN and the other assayed the leading front of migrating PMN. AHN-12.1 and KC56(T200) significantly inhibited PMN chemotaxis to LTB_4 and C5a. AHN-12.1 slightly inhibited PMN chemotaxis to FMLP, but KC56(T200) did not. In contrast, AHN-12 and HLe-1 did not significantly inhibit PMN chemotaxis to any of the chemoattractants. None of the CD45 mAb inhibited PMN chemotaxis to neutrophil-activating protein-1/IL-8. None of the CD45 mAb inhibited PMN superoxide production. These results suggest that PMN CD45 epitopes may interact with LTB_4 and C5a receptor-associated molecules and regulate chemotactic responses.

The leukocyte common Ag family (CD45) is a group of high m.w. glycoproteins that are expressed on the plasma membranes of all leukocytes. CD45 is transcribed from a single gene, and the family of proteins is generated by alternative splicing of three exons (recently reviewed in Ref. 1). The patterns of expression are controlled in a leukocyte-specific manner. PMN[3] and thymocytes express only the lowest m.w. 180-kDa, isoform. B lymphocytes express only the highest m.w., 220-kDa, isoform, whereas T lymphocytes have a complex expression of 180-kDa, 190-kDa, and 205-kDa isoforms, which change during differentiation and activation. The complete primary sequence for CD45 has been determined from analysis of cDNA clones (1). CD45 consists of an amino-terminal external domain that varies in size from 391 to 552 amino acids, a membrane-spanning region of 22 amino acids, and a cytoplasmic domain of 705 amino acids. The cytoplasmic domain contains two tandem subdomains of 300 amino acids and has protein tyrosine phosphatase activity (2). In vitro, CD45 dephosphorylates myelin basic protein, epidermal growth factor receptor, insulin receptor, and $p56^{lck}$ protein tyrosine kinases (3).

CD45 appears to regulate signal transduction and lymphocyte activation by specific association with receptor molecules on T and B lymphocytes (4, 5). CD45 may modify the function of lymphocyte receptors when brought into close physical association with them. Because the cytoplasmic portion of CD45 has protein tyrosine phosphatase activity, it is possible that molecules that interact with the cytoplasmic domain are dephosphorylated at critical tyrosine residues during lymphocyte activation.

The function of CD45 on phagocytic leukocytes remains unknown. CD45 is found on the plasma membranes of PMN and intracellularly is associated with tertiary and secondary granules (6). Agents that stimulate PMN degranulation induce an up-regulation of the 180-kDa CD45 protein on PMN plasma membranes (6, 7). As participants in the Fourth International Leukocyte Differentiation Antigen Workshop (Vienna, Austria, 1989), we screened more than 200 mAb for their effects on human PMN chemotaxis. One antibody that we added as a negative control to the CD15 Workshop Panel significantly inhibited PMN chemotaxis to LTB_4 and was classified as a CD45 mAb. This observation prompted the present investigation, in which we examined the effects of several CD45 mAb on human PMN chemotaxis to a variety of chemoattractants, including FMLP, LTB_4, re-

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[3] Abbreviations used in this paper: PMN, neutrophils; LTB_4, leukotriene B_4; NAP-1, neutrophil-activating protein-1; DFP, diisopropylfluorophosphate; NP-40, Nonidet P-40; PVDF, polyvinylidene difluoride; NGS, normal goat serum; T-TBS, Tween-Tris-buffered saline.

combinant human C5a, and recombinant human NAP-1, recently designated IL-8. Selected CD45 mAb significantly inhibited PMN chemotaxis to LTB_4 and C5a; only one mAb slightly inhibited PMN chemotaxis to FMLP, and none of the mAb inhibited PMN chemotaxis to NAP-1/IL-8. None of the mAb inhibited PMN superoxide production. These observations suggest that PMN CD45 epitopes may interact with LTB_4 and C5a receptor-associated molecules and regulate chemotactic responses.

MATERIALS AND METHODS

Antibodies and reagents. FMLP, propidium iodide, horse heart ferricytochrome *c* (type VI), bovine superoxide dismutase (type I), and cytochalasin B were purchased from Sigma Chemical Co. (St. Louis, MO). LTB_4 was purchased from Upjohn Diagnostics (Kalamazoo, MI). Recombinant human C5a was a generous gift from Pfizer (Groton, CT). Recombinant human NAP-1/IL-8 was a generous gift from Sandoz Forschungsinstitut (Vienna, Austria). [^{3}H]LTB_4 (specific activity, 196 Ci/mmol) was purchased from New England Nuclear (Boston, MA). The CD45 antibodies HLe-1 and KC56(T-200) were purchased from Becton-Dickinson (Mountain View, CA) and Coulter Immunology (Hialeah, FL), respectively. The CD15 antibody Leu-M1 and the CD16 antibody Leu-11a, fluorescein-conjugated IgG1 and IgM isotype control reagents, and goat anti-mouse IgG were purchased from Becton-Dickinson. Phycoerythrin-conjugated IgG1 isotype control was purchased from Coulter Immunology.

Immunization and production of hybridoma cell lines. AHN-12, AHN-12.1, AHN-12.2, AHN-12.3, and AHN-12.4 were produced by immunizing 8-week-old female BALB/cJ mice (The Jackson Laboratory, Bar Harbor, ME) with an i.p. injection of 50 μg of protein in CFA (GIBCO Laboratories, Grand Island, NY). The protein preparation was purified by wheat germ affinity chromatography (8) from eosinophils of a patient with hypereosinophilic syndrome. Mice were subsequently immunized s.c., at weekly intervals, with 50 μg of purified protein in IFA (GIBCO). One week after the third immunization, mice received 50 μg of protein in PBS, pH 7.2, i.p. Four days later, their spleen cells were fused with murine myeloma P3-X63-Ag8.653 cells, at a ratio of four spleen cells per myeloma cell, as previously described (9). Hybridoma cell cultures secreting antibodies that bound to PMN were twice cloned in soft agar as described (9). Ig isotype was determined by using a commercially available isotyping kit (Bio-Rad, Richmond, CA). All AHN-12 mAb preparations were heated at 56°C for 30 min before they were tested in chemotaxis assays.

^{125}I-labeling. PMN and lymphocytes were labeled with ^{125}I, by using lactoperoxidase, as described (9). Briefly, 1 to 2 × 10^7 cells were suspended in 1 ml of PBS on ice and 5 U of lactoperoxidase (Sigma) in PBS, 1 mCi of Na^{125}I (carrier-free; Amersham, Arlington Heights, IL), and 10 μl of 0.06% H_2O_2 were added. After 5 min, an additional 10 μl of 0.06% H_2O_2 were added and, 5 min later, DFP (Sigma) was added to a final concentration of 5 mM. The cells were then incubated on ice for 10 min, recovered by centrifugation at 400 × *g* for 8 min, and then washed twice with 10 ml of PBS at 0°C. All steps were performed at 0 to 4°C. All reactions involving DFP were performed in a fume hood and all articles contacting DFP were washed in 5 M NaOH before removal from the hood. Radiolabeled cells (1 to 2 × 10^7) were suspended in 1 ml of solubilization buffer (20 mM Tris, pH 7.6, 150 mM NaCl, 0.5% NP-40, 0.02% NaN_3, 2 mM PMSF and incubated on ice for 20 min. The suspensions were then centrifuged at 8800 × *g* for 30 min at 4°C, and the supernatant (extract) was used for immunoprecipitation, as described below.

Immunoprecipitation and SDS-PAGE. Immunoprecipitation was performed as previously described (9). Briefly, radiolabeled cell proteins were immunoprecipitated from reaction mixtures containing cell extract, antibody, 20 mM Tris-HCl, pH 8.2, 100 mM NaCl, 0.5% NP-40, 1 mM EDTA, 0.125 mg/ml gelatin, and 2 mM PMSF, in a total volume of 0.25 ml in 10- × 75-mm glass tubes. After the suspension was incubated overnight at 4°C, 5 μl of anti-mouse IgM (Organon Teknika, Durham, NC) were added for AHN-12.1 or 5 μl of anti-mouse IgG^{H+L} (Organon Teknika) were added for AHN-12, AHN-12.2, AHN-12.3, and AHN-12.4, and the mixture was incubated at 4°C for 1 h. Fifty microliters of 10% *Staphylococcus aureus* (Pansorbin; Calbiochem, La Jolla, CA) were then added. After 15 min at 4°C, the mixture was washed twice by addition of 1 ml of a buffer containing 20 mM Tris-HCl, pH 8.2, 1 mM EDTA, 100 mM NaCl, 0.5% NP-40, 2.5 M KCl, and 0.25 mg/ml gelatin and centrifugation at 2400 × *g* for 20 min at 4°C. The pellet was then suspended in 1 ml of 20 mM Tris-HCl, pH 8.2, transferred to Eppendorf tubes, and centrifuged at 8800 × *g* for 5 min. The precipitate was suspended in Laemmli sample buffer [62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-ME, 0.001% bromophenol blue (10)]. The samples were then incubated at 100°C for 2 min and analyzed by SDS-PAGE. Gel slabs were stained with Coomasie blue, dried, and examined by autoradiography using Kodak X-Omat XR film.

Immunoblotting. Immunoblotting was performed as previously described (11, 12). Briefly, samples were suspended in Laemmli sample buffer without 5% 2-ME and were separated by SDS-PAGE in the Laemmli buffer system (10). The gels were then equilibrated in transfer buffer (25 mM Tris-HCl, pH 8.3, 152 mM glycine, 20% methanol) for 30 min and electroblotted onto the transfer membrane (Immobilon PVDF transfer membranes; Millipore Corp., Bedford, MA) at 30 V for 12 h at 4°C and then at 80 V for 2 h at 4°C (11). Protein transfer was monitored by amido black staining (11).

After transfer of proteins onto PVDF, the strips were blocked by incubation in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, containing 0.1% Tween-20 (Sigma) and 0.04% NaN_3 (T-TBS), with 5% NGS (GIBCO), overnight at 23°C with constant rocking. After two washes with T-TBS, PVDF strips (0.3 cm wide) were incubated for 2 h at 23°C in T-TBS/5% NGS, containing 2 μl of mouse ascites per 2 ml of T-TBS/5% NGS, on a rocker platform. The first antibody was removed and the strips were washed three times with T-TBS. The strips were then incubated for 1 h at 23°C with biotinylated goat anti-mouse IgG (H and L chain specific; Organon Teknika Corp., West Chester, PA) diluted 1/1000 in T-TBS/5% NGS (2 ml/strip). The second antibody was removed, and the PVDF was washed three times in T-TBS and then incubated for 30 min with avidin-conjugated alakaline phosphatase (Organon Teknika Corp.) diluted 1/5000 in T-TBS/5% NGS (5 ml/well). After two T-TBS washes, proteins were visualized by incubation in 0.3 mg/ml nitroblue tetrazolium (Sigma), 0.2 mg/ml 5-bromo-4-chloro-3-indolylphosphate (Sigma), 100 mM NaCl, 50 mM $MgCl_2$, and 100 mM Tris-HCl, pH 9.5, for 30 min at 23°C in the dark (12, 13). The reaction was stopped by washing in distilled water, at 23°C.

In some cases, as indicated, proteins were transferred onto PVDF paper, as described above, and the paper was rinsed once in 50 mM sodium acetate, pH 4.5, and then incubated in the dark in 50 mM sodium acetate, pH 4.5, with or without 40 mM $NaIO_4$, for 1 h at 23°C. The paper was then washed three times in 50 mM sodium acetate, pH 4.5, and once in T-TBS, blocked, and then probed with various antibodies, as described above. In some cases, as indicated, proteins were transferred onto PVDF and the PVDF was incubated for 30 min at 60°C in 0.5 M Tris-HCl, pH 7.0, containing 1% SDS and 5% 2-ME, and then washed three times in T-TBS and blocked before being probed with various antibodies as described above.

Isolation of human PMN. PMN were isolated from blood anticoagulated with acid citrate dextrose solution A (25 ml, containing 22.0 g/liter $Na_3C_6H_5O_7 \cdot H_2O$, 8.0 g/liter citric acid, 24.5 g/liter dextrose, per 100 ml of blood) by a modified procedure of Boyum (14). A PMN-rich plasma fraction was first obtained by a dextran sedimentation procedure, in which 3 ml of 5% dextran solution (pyrogen free, m.w. 100,000 to 200,000; U.S. Biochemical Corp., Cleveland, OH) were added per 10 ml of blood. The PMN-rich fraction was then layered over Ficoll-Hypaque (lymphocyte separation medium; Organon Teknika) as described (14). Residual RBC were removed by hypotonic lysis with sterile water for injection (sterile water for injection, USP; Abbott Laboratories, North Chicago, IL). Cell preparations contained >95% PMN that were ≥98% viable, as assessed by propidium iodide exclusion (15).

Antibody binding. All mAb preparations were centrifuged for 20 min at 7000 × *g* before use, to remove mAb aggregates. PMN were exposed to saturating concentrations of mAb at 4°C for 30 min. The cells were then gently pelleted at 100 × *g* for 7 min, the supernatant was removed, and PMN were resuspended to a concentration of 1 × 10^6 PMN/ml in HBSS containing 0.2% BSA. Saturating concentrations of mAb were determined by flow cytometric analysis. The mAb-treated PMN were evaluated by flow cytometry and in chemotaxis assays described below.

In some experiments, PMN were preexposed to FMLP (10^{-7} M), LTB_4 (10^{-8} M), C5a (10^{-7} M), or NAP-1/IL-8 (10^{-7} M) for 15 min at 4°C. Designated concentrations of fluorescently conjugated CD45 mAb were added to the PMN containing chemoattractants, and the cells were incubated with mAb and chemoattractants for an additional 30 min at 4°C. PMN were gently pelleted and resuspended in cold PBS for flow cytometric analysis.

Flow cytometry. Flow cytometric evaluations were performed with an Ortho Spectrum III flow cytometer equipped with an argon-ion laser and a model 2140 computer. At least 10,000 cells were analyzed for each sample. Results are presented as mean channel fluorescence of linear scale fluorescence intensity.

Chemotaxis assays. Chemotaxis was assayed in a multiwell chemotaxis chamber as described (16). Cells were suspended in HBSS/BSA at a concentration of 1 × 10^6 PMN/ml. Assays were

performed in which the total number of PMN migrating through polyvinylpyrrolidone-free polycarbonate membranes (5-μm pores; Nuclepore Corp., Pleasanton, CA) was quantified (17). Chambers were incubated for 35 min at 37°C in humid air. Chemoattractants were diluted in HBSS/BSA. After incubation, nonmigrated PMN were wiped off the filters, and the filters were fixed in methanol and stained with Diff-Quik (Dade Diagnostics, Inc., Aguada, Puerto Rico). Migrated PMN were counted with an Optomax System IV image analyzer (Optomax, Inc., Hollis, NH). Random migration was determined by quantifying PMN migration to medium alone. The chemokinetic migration (chemokinesis) was assayed by placing equal concentrations of the chemoattractants in the upper and lower wells of the chamber. Triplicate determinations of the total number of migrating PMN/mm^2 of filter surface were performed for each PMN donor; five or six different PMN donors were tested.

PMN chemotaxis leading front analyses (18) were performed in some experiments with the multiwell chamber and cellulose nitrate filters (5-μm pores; Sartorius, Gottingen, West Germany). PMN (1×10^6/ml in HBSS/BSA) were incubated in chemotaxis chambers for 45 min at 37°C in humid air. Filters were fixed and stained as described (19). The leading front was determined by measuring the distance traveled from the upper surface of the filter to the position where the three farthest migrating PMN were found (18). Chemotaxis assays were performed in triplicate and the values represent the means of five measurements of each triplicate (15 fields). Statistical analyses were performed using the two-tailed Student's *t*-test.

Separation of chemotactically responsive and nonresponsive PMN. Migrating and nonmigrating PMN were isolated with a chemotaxis separation chamber described previously (20, 21) (Neuro Probe, Inc., Cabin John, MD). PMN were removed from the upper (chemotactically nonresponsive PMN) and lower (chemotactically responsive PMN) membrane surfaces by gentle washing of the membrane surfaces with cold Ca^{2+}- and Mg^{2+}-free HBSS/BSA. Isolated PMN were adjusted to a concentration of 1×10^6 PMN/ml and maintained at 4°C. In some experiments, PMN were fixed with 3.7% formalin before mAb staining; in other experiments, PMN were not fixed and remained viable during mAb staining at 4°C.

[^{3}H]LTB$_4$ binding to PMN. [^{3}H]LTB$_4$ binding to PMN was determined as described (22). Preliminary experiments were performed to determine the approximate half-maximal binding concentration. Subsequent experiments were performed in which PMN were incubated with saturating concentrations of designated mAb for 30 min at 4°C. PMN were gently pelleted at $100 \times g$ for 7 min and the supernatants were removed. PMN were resuspended in HBSS containing 0.1% OVA. Reactions containing 1×10^6 PMN and ~5 nM [^{3}H]LTB$_4$, with or without 1000-fold excess unlabeled LTB$_4$, were incubated in a final volume of 200 μl for 30 min at 4°C. Reaction mixtures were layered onto 600 μl of silicone oil (General Electric SF 1250 silicone fluid; Harwick Chemical Corp., Trenton, NJ) and centrifuged for 90 s in 1.5-ml microfuge tubes in a microcentrifuge (Brinkmann Instruments, Inc., Westbury, NY). Samples were processed for counting as previously described (23). Nonspecific binding was <15% of the total binding. Results are reported as the means ± 1 SD of quadruplicate replicates.

Fluorescence microscopy. PMN were analyzed for CD45 distribution with a laser scanning confocal microscope [Bio-Rad MRC-500 laser scanning system (Cambridge, MA), connected to a Nikon Microphot-FX microscope]. In some experiments, 100 μl of PMN (1×10^6/ml) were pipetted into individual blind well chemotaxis chambers (Neuro Probe, Inc.) containing HBSS/BSA medium or optimal chemotactic concentrations of chemoattractants in the lower wells and 13-mm-diameter polycarbonate membranes with 5-μm pores. Conditions for the chemotaxis assay were identical to those described above for the multiwell microchemotaxis assay. After a 35-min incubation at 37°C, the filters were removed and fixed in 3.7% formalin-PBS. The filters were washed several times with PBS before staining with mAb. After staining, filters were washed with PBS and mounted with Gel-mount medium (Biomeda Corp., Foster City, CA). In other experiments, control (without mAb) or CD45 mAb-treated PMN were plated in glass Lab-Tek chambers (Nunc Inc., Naperville, IL) and incubated for 15 min at 37°C to allow PMN to settle and adhere, and then PMN were stimulated with 10^{-7} M FMLP, 10^{-8} M C5a, or 10^{-7} M LTB$_4$ for periods of 5 s to 15 min. The cells were fixed with 3.7% formalin-PBS and washed with PBS before staining with mAb. After staining, the chambers were disassembled and the specimens were mounted with Gel-mount medium. Images were photographed from a high resolution, flat screen, black and white monitor (VM1710; Lucius & Baer, Geretsried, W. Germany) with Kodak Plus-X pan film.

Superoxide assay. PMN superoxide production was assayed by a modified procedure of Babior *et al.* (24). PMN were preincubated with or without the CD45 mAb, isotype control mAb, Leu-M1, or Leu-11a mAb, for 30 min at 4°C, as described above in *Antibody binding*. Reaction mixtures containing 1×10^6 PMN in complete HBSS with 0.1 mM ferricytochrome *c* (Type VI) and 5 μg/ml cytochalasin B were preincubated for 10 min at 37°C before stimuli addition. Reference cuvettes contained the same components and 60 μg/ml superoxide dismutase (type I). Superoxide production was quantified from unstimulated PMN and PMN stimulated with 1 μM FMLP, 1 μM LTB$_4$, and 0.1 μM C5a. The extinction coefficient of 20,000 M^{-1} cm^{-1} at 550 nm was used for cytochrome *c* (25). Results are reported as the mean ± 1 SD of duplicate determinations from two separate experiments with different PMN donors and are expressed as nmol of superoxide/1×10^6 PMN/10 min.

RESULTS

Characterization of mAb. Eleven mAb were initially evaluated in this study: seven mAb that recognize CD45 Ag, two control mAb that recognize the myeloid Ag CD15 and CD16, included as isotype controls, and two isotype controls that do not bind to myeloid cells. A summary of the mAb, their respective isotypes, and their binding properties to PMN are presented in Table I. In flow cytometric analyses, the CD45 mAb bound homogeneously to 100% of human PMN (Fig. 1).

AHN-12, AHN-12.2, AHN-12.3, and AHN-12.4 were classified at the Fourth International Workshop on Leukocyte Differentiation Antigens (Vienna, Austria) as CD45 mAb [Nonlineage Workshop Panel mAb numbers

TABLE I
mAb evaluated

Name	Isotype	CD	PMN Binding
Isotype Control	IgG1	None	–
Isotype Control	IgM	None	–
Leu-M1	IgM	CD15	+ (100%)
Leu-11a	IgG1	CD16	+ (100%)
AHN-12	IgG1	CD45	+ (100%)
AHN-12.1	IgM	CD45	+ (100%)
AHN-12.2	IgG1	CD45	+ (100%)
AHN-12.3	IgG1	CD45	+ (100%)
AHN-12.4	IgG1	CD45	+ (100%)
HLe-1	IgG1	CD45	+ (100%)
KC56	IgG1	CD45	+ (100%)

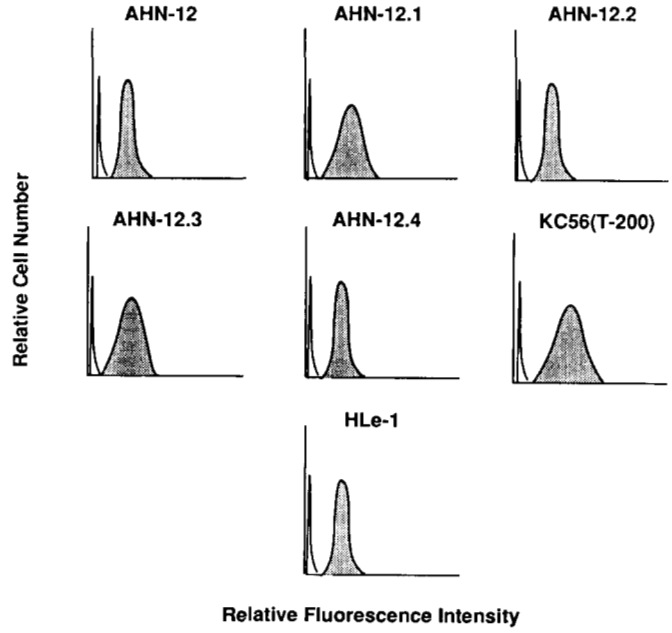

Figure 1. Representative flow cytometric histogram profiles of CD45 mAb binding to human PMN. *Vertical axis*, relative cell number; *horizontal axis*, relative linear fluorescence intensity. *Shaded histograms*, CD45 mAb binding; *unshaded histograms*, isotype control binding.

N810, N809, N88, and N811, respectively (26)]. The AHN-12 mAb were all prepared by immunizing mice with protein purified by wheat germ affinity chromatography from eosinophils of a patient with hypereosinophilic syndrome, as described in *Materials and Methods.* Immunoprecipitation studies using extracts of surface-labeled PMN and lymphocytes were performed to confirm the specificity of the AHN-12 mAb. Results illustrating the specificity of AHN-12.1 are shown in Figure 2. AHN-12.1 immunoprecipitated a ~180-kDa surface-labeled protein from PMNs (Fig. 2, *lane A*), whereas normal mouse serum (Fig. 2, *lane B*) did not. AHN-12.1 immunoprecipitated four surface-labeled proteins of ~180, 190, 205, and 220 kDa from lymphocytes (Fig. 2, *lane C*), whereas normal mouse serum (Fig. 2, *lane D*) did not. The other AHN-12 mAb exhibited similar immunoprecipitation patterns (26). The flow cytometric binding profiles and immunoprecipitation patterns of the AHN-12, AHN-12.1, AHN-12.2, AHN-12.3, and AHN-12.4 mAb were characteristic of CD45 mAb.

Effects of reduction and periodate on Ag detection. When PMN proteins were separated by SDS-PAGE under nonreducing conditions before transfer to PVDF, antibodies AHN-12 to AHN-12.4 each detected a 180-kDa protein by immunoblotting, identical to the pattern in Figure 2, *lane A.* In contrast, when PMN membrane proteins were separated by SDS-PAGE under reducing conditions before immunoblotting with AHN-12 to AHN-12.4, the 180-kDa protein was no longer detected. Similarly, when proteins were separated by SDS-PAGE under nonreducing conditions, transblotted onto PVDF, incubated in 0.5 M Tris-HCl, pH 7.0, containing 1% SDS and 5% 2-ME, at 60°C for 30 min, and then probed with mAb AHN-12 to AHN-12.4 and developed as described in *Materials and Methods,* no protein bearing the CD45 Ag could be detected. To determine the role of carbohydrates in the epitopes recognized by AHN-12 to AHN-12.4, proteins were transferred onto PVDF and incubated for 1 h with 40 mM $NaIO_4$ in 50 mM sodium acetate buffer, pH 4.5. Treatment of the transferred membrane proteins with $NaIO_4$ also markedly decreased or eliminated the ability of AHN-12 to AHN-12.4 to detect the CD45 Ag.

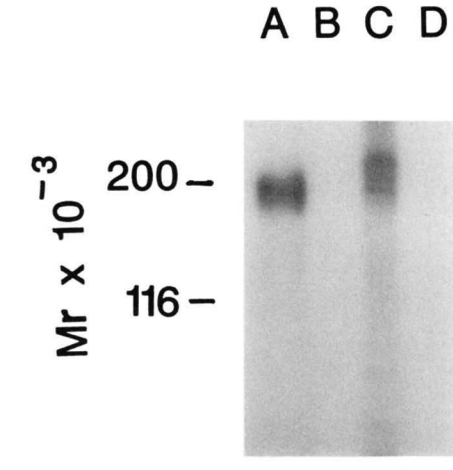

Figure 2. Immunoprecipitation and PAGE of ^{125}I-labeled PMN and lymphocyte surface proteins. PMN (*lanes A* and *B*) and lymphocytes (*lanes C* and *D*) were labeled at the cell surface (1 to 2×10^7 cells) with ^{125}I, solubilized, immunoprecipitated with AHN-12.1 (*lanes A* and *C*) or normal mouse serum (*lanes B* and *D*), and analyzed by SDS-PAGE and autoradiography as described in *Materials and Methods.* Proteins used as molecular weight standards were: myosin heavy chain, 200,000, and *Escherichia coli* β-galactosidase, 116,000.

The sensitivity of the epitopes recognized by AHN-12 to AHN-12.4 to both reduction and periodate treatment suggests that, although carbohydrates are important in the structure recognized by these antibodies, the tertiary structure of the protein backbone is also probably an important component of these epitopes.

Effects of mAb on PMN chemotaxis. The panel of mAb was initially screened for effects on PMN chemotaxis to FMLP and LTB_4 in two separate chemotaxis assays. Each assay provided unique quantitative information about the effects of mAb on PMN chemotaxis. One assay quantified the total number of PMN migrating through 10-μm-thick polycarbonate membranes (total migration response), and the second assay measured the distance migrated by the fastest migrating PMN (leading front). PMN from five or six different PMN donors were evaluated with the panel of mAb in separate experiments, in which triplicate assays were performed with each mAb and chemoattractant. The effects of mAb on chemotactic responses to FMLP (5×10^{-8} M) and LTB_4 (1×10^{-8} M) are summarized as the percentage of the isotype control response (Fig. 3). The majority of CD45 mAb did not inhibit PMN chemotaxis to FMLP (Fig. 3, *A* and *B*), whereas most of the CD45 mAb inhibited PMN chemotaxis to LTB_4 (Fig. 3, *C* and *D*). Only mAb AHN-12.1 had a consistent significant inhibitory effect on PMN chemotaxis to FMLP (Fig. 3*A*); however, the inhibition was not marked. The CD16 and CD15 mAb, Leu 11a and Leu M1, and the CD45 mAb, HLe-1 and AHN-12, did not inhibit chemotaxis to either LTB_4 or FMLP. In general, both of the chemotaxis assays correlated closely; mAb that inhibited the total number of migrating PMN also inhibited the PMN leading front. The only exception was mAb AHN-12.1; AHN-12.1 had a slight but significant inhibitory effect on PMN chemotaxis to FMLP in the polycarbonate assay (Fig. 3*A*); however, the slight inhibitory effect in the leading front assay for chemotaxis to FMLP was not significant (Fig. 3*B*). CD45 mAb that affected PMN chemotaxis had an inhibitory effect over a broad chemoattractant concentration range (Fig. 4*A*). AHN-12.1 and KC56 significantly inhibited PMN chemotaxis over the LTB_4 concentration range of 1 nM to 1 μM ($p < 0.02$), without shifting the concentration response. CD45 mAb did not alter the chemotactic concentration response of PMN to FMLP (Fig. 4*B*). None of the mAb significantly affected PMN random migration or chemokinesis. The combination of random migration and chemokinetic migration responses accounted for 25% of the total migration responses.

Identical chemotaxis experiments were performed with a selected panel of CD45 mAb and the isotype control mAb, with recombinant human C5a (1×10^{-8} M) and recombinant human NAP-1/IL-8 (1×10^{-8} M) as the chemoattractants (Fig. 5). AHN-12.1 and KC56 significantly inhibited PMN chemotaxis to C5a, whereas AHN-12, HLe-1, and the CD16 and CD15 mAb did not (Fig. 5, *A* and *B*). In contrast, none of the mAb significantly inhibited PMN chemotaxis to NAP-1/IL-8 (Fig. 5, *C* and *D*).

CD45 mAb exhibited different effects on PMN chemotaxis (summarized in Table II). AHN-12.1 and KC56 consistently inhibited chemotaxis to LTB_4 and C5a. AHN-12.1 was the only CD45 mAb that slightly but significantly inhibited chemotaxis to FMLP. None of the mAb

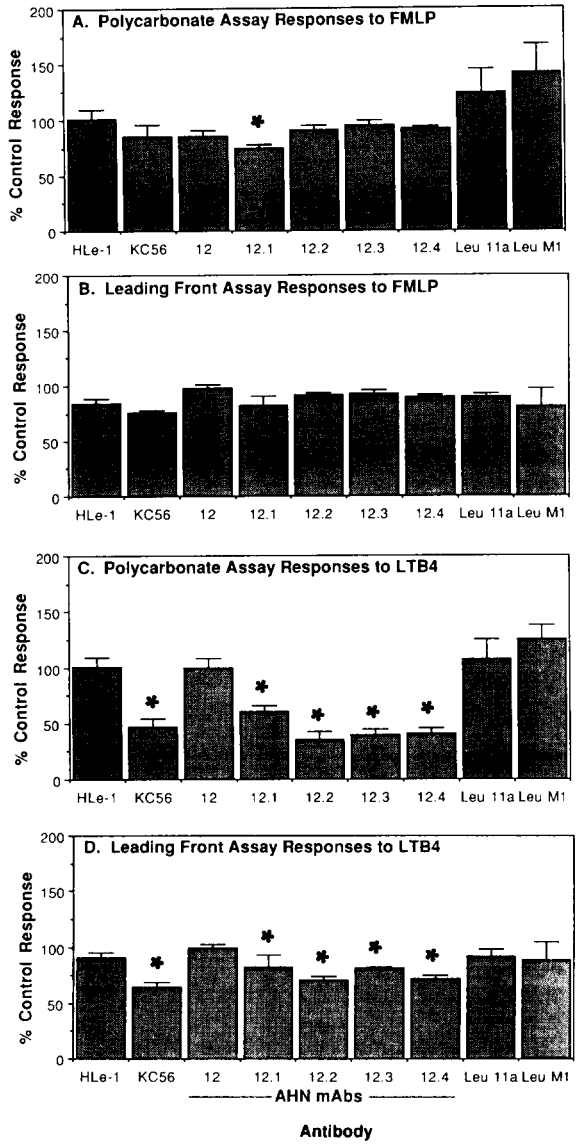

Figure 3. Effects of mAb on PMN migration to FMLP (5×10^{-8} M) and LTB_4 (1×10^{-8} M) in the polycarbonate membrane and leading front chemotaxis assays. PMN chemotaxis to FMLP in the polycarbonate assay (*A*) and leading front assay (*B*) and PMN chemotaxis to LTB_4 in the polycarbonate assay (*C*) and leading front assay (*D*) were evaluated with PMN from five or six different donors. Results are expressed as the percentage of control response (isotype control) and are presented as the means ± 1 SEM of five or six experiments. *, Significant effect ($p < 0.02$). The sum of the PMN random migration and chemokinetic migration accounted for ~25% of the total migratory response.

significantly inhibited chemotaxis to NAP-1/IL-8. Flow cytometric analyses were performed on all CD45 mAb-treated PMN preparations, to confirm that mAb remained associated with PMN. In each chemotaxis experiment (Figs. 3 and 5), aliquots of mAb-treated PMN were removed and held at either 4°C or 37°C for 35 min (the chemotaxis assay period). Samples held at 4°C and 37°C exhibited fluorescence histogram profiles identical to those illustrated in Figure 1, and all of the CD45 mAb remained associated with PMN after the 35-min incubation.

Competitive binding of chemoattractants and CD45 mAb. Experiments were performed to determine whether CD45 mAb and chemoattractants competitively inhibited each other's binding. PMN were pretreated with saturating concentrations of FMLP (1×10^{-7} M), LTB_4 (1×10^{-8} M), C5a (1×10^{-7} M), or NAP-1/IL-8 (1×10^{-7} M), as described in *Materials and Methods*, and PMN were evaluated with flow cytometry for CD45 mAb binding. Chemoattractant binding did not affect CD45 mAb binding (Fig. 6*A*). The same experiments were performed with HLe-1 and AHN-12.1, and results were identical to those presented for KC56 in Figure 6A. PMN pretreated with CD45 mAb were evaluated for [^{3}H]LTB_4 binding, as described in *Materials and Methods*. [^{3}H]LTB_4 binding was not significantly inhibited ($p > 0.05$) when PMN were pretreated with saturating concentrations of CD45 mAb (Fig. 6*B*).

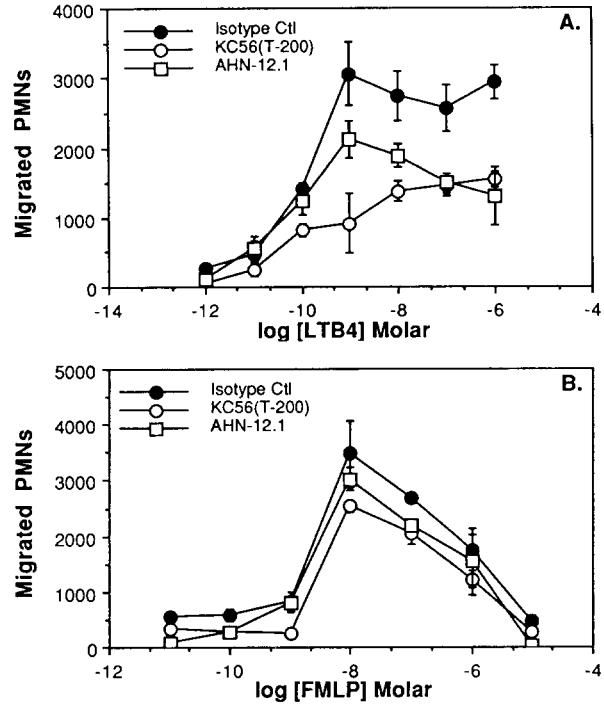

Figure 4. Effects of mAb on PMN chemotaxis concentration responses to LTB_4 and FMLP in the polycarbonate assay. PMN were exposed to isotype control mAb (●), KC56 (○), or AHN-12.1 (□) and tested for chemotactic responsiveness to a range of LTB_4 (*A*) or FMLP (*B*) concentrations. Data are presented as the means ± 1 SD of migrated PMN/mm^2 of filter surface of triplicate assays from an individual PMN donor. The experiment was repeated with PMN from two additional donors and similar results were obtained. The random migration responses were 430 ± 80 PMN/mm^2 of filter surface.

CD45 expression on chemotactic and nonchemotactic PMN. Untreated PMN were exposed to FMLP (1×10^{-7} M) or LTB_4 (1×10^{-8} M) in chemotactic separation chambers (21, 22) for 35 min at 37°C. The chemotactically responsive (migrators) and nonresponsive (nonmigrators) PMN were isolated and evaluated for CD45 expression by quantification of fluorescent CD45 mAb binding with flow cytometry. Both PMN populations continued to express CD45 after exposure to chemoattractant gradients (Table III). Control PMN suspensions incubated for 35 min at 37°C slightly up-regulated CD45, when compared with control PMN continuously maintained at 4°C for 35 min. The chemotactically responsive PMN slightly down-regulated CD45 after migration, whereas the nonchemotactic PMN did not exhibit a change in CD45 expression, when compared with the 37°C control PMNs. The CD45 expression on chemotactically responsive PMN was significantly lower than on the 37°C control PMN ($p < 0.05$) only when PMN had migrated to LTB_4 and were subsequently stained with fluorescent KC56. PMN that mi-

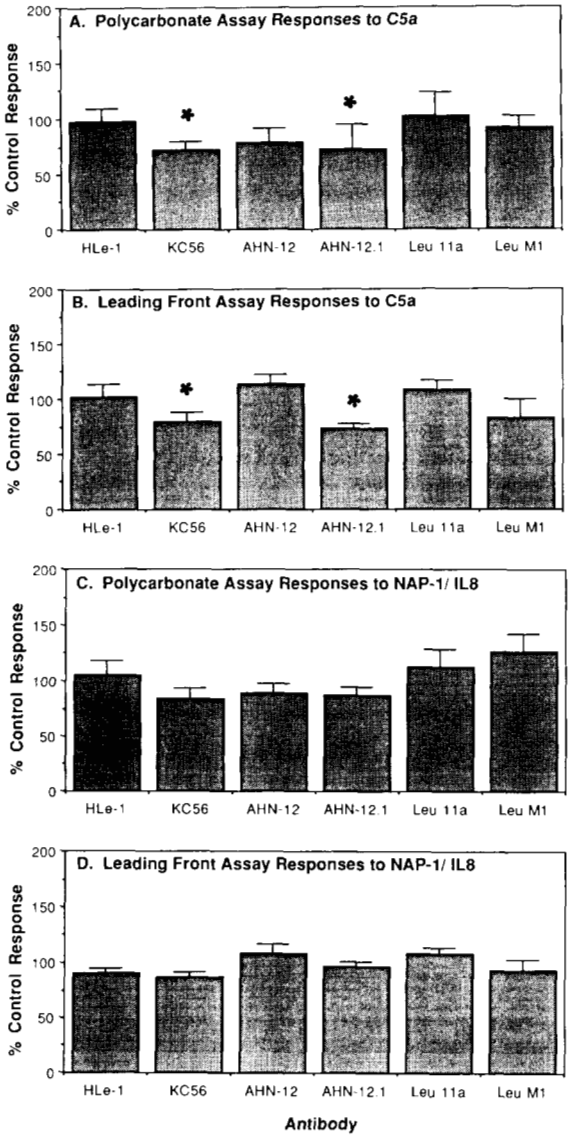

Figure 5. Effects of mAb on PMN migration to recombinant human C5a (1×10^{-8} M) and NAP-1/IL-8 (1×10^{-8} M) in the polycarbonate membrane and leading front chemotaxis assays. PMN chemotaxis to C5a in the polycarbonate assay (*A*) and leading front assay (*B*) and PMN chemotaxis to NAP-1/IL-8 in the polycarbonate assay (*C*) and leading front assay (*D*) were evaluated with PMN from five or six different donors. Results are expressed as the percentage of control response (isotype control) and are presented as the means ± 1 SEM of five or six experiments. *, Significant effect ($p < 0.02$). The sum of the PMN random migration and chemokinetic migration accounted for ~25% of the total migratory response.

TABLE II

Summary of inhibitory effects of CD45 antibodies on PMN chemotaxis[a]

Chemoattractant	Antibody			
	AHN-12	AHN-12.1	HLe-1	KC56
FMLP	–[b]	↓[c]	–	–
LTB_4	–	↓	–	↓
C5a	–	↓	–	↓
NAP-1/IL-8	–	–	–	–

[a] Data are summarized from the polycarbonate chemotaxis experiments, in which five or six different PMN donors were evaluated for each mAb.

[b] –, No significant effect.

[c] ↓, Significant inhibition ($p < 0.02$).

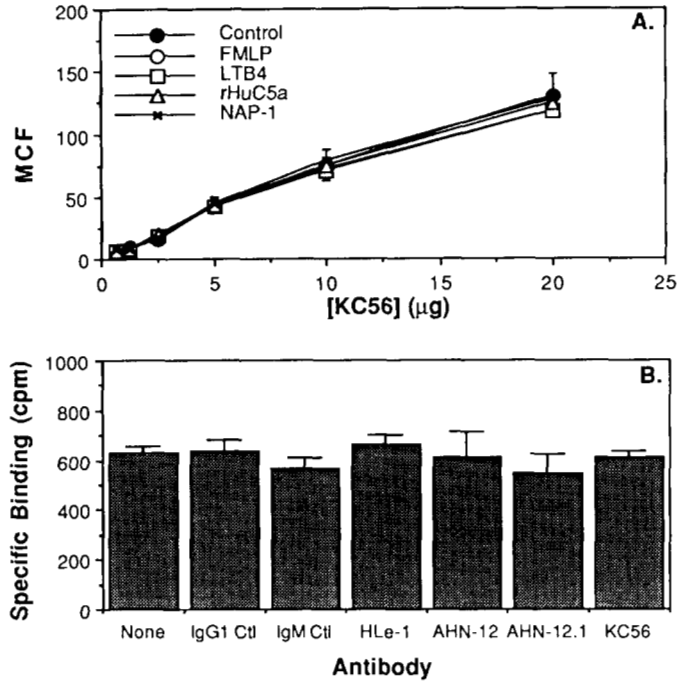

Figure 6. Competitive binding of chemoattractants and CD45 mAb. *A*, PMN were either untreated (●) or pretreated with saturating concentrations of FMLP (○), LTB_4 (□), C5a (△), or NAP-1/IL-8 (×) and then evaluated for fluorescent CD45 mAb binding as described in *Materials and Methods*. Data are presented as the mean channel fluorescence (*MCF*) values ± 1 SD of PMN for the indicated concentrations of KC56 (*μg/ml*). *B*, 1×10^6 PMN were pretreated with saturating concentrations of CD45 mAb or equivalent concentrations of isotype control mAb, at 4°C for 30 min, and then exposed to an approximate half-maximal binding concentration of 5 nM [^{3}H]LTB_4 for 30 min at 4°C. Results are presented as the mean specific cpm bound ± 1 SD of quadruplicate replicates from an individual PMN donor. [^{3}H]LTB_4 binding to CD45 mAb-treated PMN was not significantly different than binding to isotype control-treated PMN ($p > 0.05$). The experiment was repeated with a different donor and identical results were obtained.

grated to FMLP did not demonstrate significantly decreased binding of KC56, when compared with 37°C controls. Binding of HLe-1 was not significantly decreased on chemotactically responsive PMN to either chemoattractant (Table III).

PMN on polycarbonate membranes from chemotaxis chamber assays were fixed and stained with fluorescent CD45 mAb and analyzed by fluorescence microscopy. PMN on the upper membrane surface (nonmigrators; Fig. 7*A*) and PMN on the lower membrane surface (migrators; Fig. 7*B*) exhibited similar CD45 distribution patterns. CD45 remained diffusely expressed on the external PMN plasma membranes. Some regions of cells exhibited punctate patches of CD45; however, the patches did not consistently polarize in discrete regions of the PMN. Identical results were obtained when either control or CD45 mAb-treated PMN were plated on glass surfaces and exposed to 10^{-7} M FMLP, 10^{-8} M LTB_4, or 10^{-7} M C5a for periods of 5 s to 15 min. The CD45 distribution patterns were identical to those in Figure 7. Neither PMN CD45 capping nor internalization was detected during the 5-s to 15-min exposures to chemoattractants, using fluorescent microscopic analysis.

Effects of mAb on PMN superoxide production. None of the mAb affected superoxide production of resting or LTB_4-, C5a-, or FMLP-stimulated PMN. Control (without mAb pretreatment) resting PMN released 1.5 ± 0.5 nmol of superoxide/1×10^6 PMN, LTB_4-stimulated PMN re-

TABLE III

CD45 expression on chemotactically responsive and nonresponsive PMN

CD45 mAb	Mean Channel Fluorescence[a]							
	FMLP-Exposed PMN[b]				LTB_4-Exposed PMN			
	4°C Control	37°C			4°C Control	37°C		
		Control	Migrators	Nonmigrators		Control	Migrators	Nonmigrators
KC56[c]	73 ± 19	96 ± 24	70 ± 23	83 ± 20	86 ± 13	107 ± 16	54 ± 13[e]	80 ± 13
HLe-1[d]	43 ± 7	48 ± 8	38 ± 6	46 ± 8	41 ± 7	53 ± 8	40 ± 7	50 ± 8

[a] Mean channel fluorescence values ± 1 SD of six replicates from two separate experiments for each condition. Isotype control values were identical for each condition evaluated (mean channel fluorescence, 10 ± 1).

[b] PMN suspensions were exposed to chemoattractant for 35 min at either 4°C or 37°C. PMN on the polycarbonate membranes in chemotactic separation chambers were exposed to a gradient of chemoattractant at 37°C for 35 min; chemotactic (migrators) and nonchemotactic PMN (nonmigrators) were then isolated. PMN were washed and stained with CD45 mAb.

[c] KC56 mAb was phycoerythrin conjugated.

[d] HLe-1 mAb was fluorescein conjugated.

[e] Mean channel fluorescence is significantly less than the corresponding 37°C control and the 4°C control values ($p < 0.05$). This condition was the only condition that demonstrated a significant difference from corresponding control values.

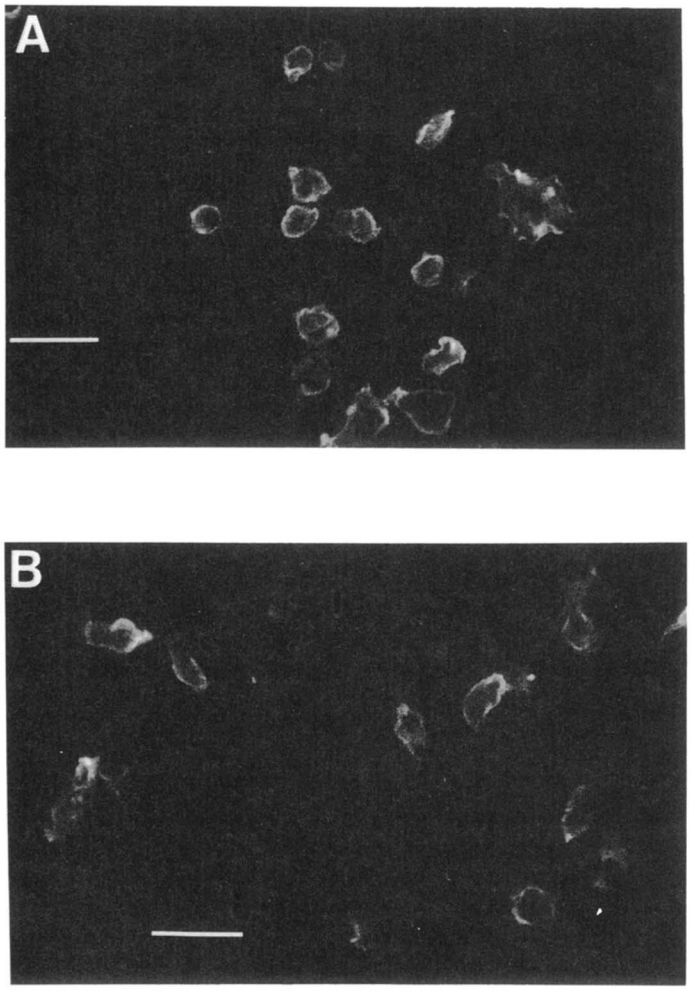

Figure 7. CD45 distribution on nonchemotactic and chemotactic PMN. PMN on the upper membrane surface of polycarbonate membranes in a chemotaxis chamber assay (*A*) did not migrate in response to LTB_4. PMN on the lower polycarbonate membrane surface (*B*) migrated through the membrane pores in response to LTB_4. PMN shown in *A* and *B* were fixed as described in *Materials and Methods* and stained with phycoerythrin-conjugated KC56. Identical results were obtained with all of the CD45 mAb. Both PMN populations exhibited similar distributions of CD45 on their external plasma membranes. *Bar*, 10 μm.

leased 2.5 ± 0.5 nmol of superoxide/1 × 10^6 PMN, C5a-stimulated PMN released 7.0 ± 1.0 nmol of superoxide/1 × 10^6 PMN, and FMLP-stimulated PMN released 18.5 ± 3.5 nmol of superoxide/1 × 10^6 PMN. All isotype control-, CD45-, Leu-M1-, and Leu-11a-mAb-treated PMN superoxide responses were identical. Superoxide values for resting and LTB_4-, C5a-, or FMLP-stimulated PMN pretreated with mAb were 2.0 ± 0.5, 3.0 ± 0.5, 7.5 ± 1.2, and 19.5 ± 4.0 nmol of superoxide/1 × 10^6 PMN, respectively. None of the mAb stimulated PMN superoxide production of resting PMN over a 35-min incubation period at 37°C. All superoxide values of mAb-treated resting PMN were ≤2.0 ± 0.5 nmol/1 × 10^6 PMN.

DISCUSSION

The results of this study demonstrate that mAb binding to selected epitopes of CD45 has a significant inhibitory effect on human PMN chemotaxis to LTB_4 and C5a. The CD45 mAb evaluated in this study did not affect PMN chemotaxis to NAP-1/IL-8, and only one mAb (AHN-12.1) had a slight but significant inhibitory effect on chemotaxis to FMLP. Because the CD45 mAb bound and remained associated with PMN but did not uniformly inhibit chemotaxis to LTB_4 and C5a, it appears that certain epitopes of CD45 are important in PMN chemotactic responses. The mechanism by which some CD45 mAb inhibit chemotaxis remains to be determined. One possibility is that LTB_4 and C5a receptor-associated molecules may directly interact with specific CD45 epitopes during chemotaxis. Binding mAb to critical CD45 epitopes may sterically block the interaction of CD45 with receptor-associated molecules. Another possibility is that mAb binding to critical CD45 epitopes may either activate or inactivate the protein tyrosine phosphatase activity of the cytoplasmic CD45 domains. Alterations of the phosphatase activity may be important in PMN chemotaxis to specific chemoattractants.

The effects of CD45 mAb on PMN chemotaxis to LTB_4 were not due to competitive inhibition of LTB_4 binding to PMN receptors. Pretreatment of PMN with saturating concentrations of CD45 mAb did not inhibit [^{3}H]LTB_4 binding to PMN. These results indicate that, if CD45 epitopes interact with LTB_4R or receptor-associated molecules, they interact at determinants distinct from the LTB_4 ligand-binding domain. It is unlikely that CD45 is a receptor for LTB_4, C5a, FMLP, or NAP-1/IL-8. The m.w. for the LTB_4R has been postulated to be 60 kDa (27), and a m.w. range of 48 to 52 kDa has been proposed for the C5aR (28–30), whereas the CD45 molecule has a m.w. of ~180 kDa in PMN. The recently cloned FMLPR is a distinct membrane protein containing 350 amino acids (31) and is not related to CD45. In addition, the results pre-

sented in the present study demonstrate that pretreatment of PMN with saturating concentrations of chemoattractants did not affect the binding of CD45 mAb to PMN.

Human PMN exhibit chemotactic heterogeneity *in vitro*. Chemotactically responsive and nonresponsive subpopulations have been separated and characterized (21). The results of this study demonstrate that both PMN subpopulations continued to express CD45 on their external plasma membranes after exposure to chemoattractant gradients. Chemotactically responsive PMN slightly down-regulated CD45 after migration, whereas nonchemotactic PMN and 37°C control PMN exhibited nearly identical CD45 expression. Warming PMN from 4°C to 37°C slightly up-regulated CD45 expression. The warming effect may be related to the fusion of granule membranes with PMN plasma membranes. PMN CD45 has been intracellularly associated with tertiary and secondary granules (6, 7). Lacal *et al.* (6) have reported that PMN pretreated with cytochalasin B and stimulated with degranulating agents up-regulate PMN CD45 expression. In the present study, PMN did not up-regulate CD45 when stimulated with chemoattractants, probably because they were not treated with cytochalasin B. PMN were not cytochalasin treated in chemotaxis experiments because cytochalasin B inhibits PMN chemotaxis (32). The slight down-regulation of CD45 on chemotactically responsive PMN may be related to shedding of membrane Ag during migration or internalization of Ag during the plasma membrane and cytoskeletal reorganization that accompanies chemotaxis. Significantly decreased CD45 binding was observed only in PMN that had migrated to LTB_4 and were stained with KC56. This finding is interesting, because the KC56 mAb significantly inhibited PMN chemotaxis to LTB_4, but not FMLP. The decreased binding of KC56 to PMN that have migrated to LTB_4 may be due to an association of the CD45 epitope recognized by KC56 with other PMN surface molecules. The association of CD45 with membrane molecules during migration may reduce the availability of the epitope for recognition by KC56. HLe-1, which did not inhibit PMN chemotaxis to any of the chemoattractants, did not exhibit significantly decreased binding to chemotactically responsive PMN. CD45 remained diffusely distributed on all adherent PMN in chemoattractant gradients. Focal patches of CD45 were observed by fluorescence microscopy; however, the CD45 did not consistently localize to discrete regions of the plasma membrane. These results demonstrate that the inhibitory effects of CD45 mAb are not due to an extensive capping of CD45 on PMN plasma membranes.

Two of the control mAb in this study, the CD16 mAb Leu-11a and the CD15 mAb Leu-M1, were selected for the test panel because they bind homogeneously to normal human PMN, they do not inhibit PMN chemotaxis, and they serve as isotype-matched controls for the CD45 mAb of the panel. CD16 mAb recognize a membrane protein of 50 to 70 kDa that is associated with the IgG Fc receptor III on NK cells and PMN (33, 34). CD15 mAb recognize the sugar lacto-*N*-fucopentaose III (X-antigen) found on several PMN membrane glycoproteins of ~105, 135, 165, 185, and 220 kDa, a distinct ~180-kDa phosphotyrosine-containing protein, and several glycolipids (9, 35–42). Most CD15 mAb do not affect PMN chemotaxis, but they significantly inhibit PMN phagocytosis (36, 43). The CD15 Workshop Panel of the Fourth International Workshop on Leukocyte Differentiation Antigens (Vienna, Austria, 1989) contained the CD45 mAb AHN-12.1 of the present study [referred to as M426 in the CD15 Workshop Panel (36)] as a control mAb. In contrast to CD15 mAb, AHN-12.1 did not affect PMN phagocytosis of opsonized *S. aureus* (36), suggesting that CD45 may not be involved in phagocytosis.

The biologic ligand for CD45 remains unknown. Functional studies of CD45 have focused on lymphocyte activation and characterization of the molecules that interact with CD45 in lymphocytes. CD45 has been reported to associate with CD2 and the human MHC class I protein on human T lymphocytes (5) and with the cytoskeletal protein fodrin in a murine T lymphoma cell line (44). It is not known whether CD45 chemically or functionally modifies these molecules when associated with them. Recent studies with mutant murine T cell lines have indicated that CD45 may play a role in lymphocyte growth regulation (45, 46). CD45 appears to have the potential of interacting with a variety of leukocyte proteins and functioning as a protein tyrosine phosphatase.

The present study demonstrates that CD45 epitopes are involved in human PMN chemotaxis to certain classes of chemoattractants. None of the mAb tested in this study affected PMN superoxide production in response to LTB_4, C5a, or FMLP. These findings suggest that CD45 is not involved in PMN superoxide production. It is also possible that cytochalasin B pretreatment of PMN, which is necessary for detection of significant superoxide production from chemoattractant-stimulated PMN, may have altered the effects of CD45 mAb on superoxide production. Characterization of PMN proteins that interact with CD45 and their state of tyrosine phosphorylation during stimulation with chemoattractants should provide interesting clues to the biochemical function of CD45 in chemotaxis. It will be of interest to determine whether CD45 mAb affect other responses elicited by chemoattractant stimuli.

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