A MONOCLONAL ANTIBODY TO A HUMAN NEUTROPHIL-SPECIFIC PLASMA MEMBRANE ANTIGEN

Effect of the Antibody on the C3bi-mediated Adherence by Neutrophils and Expression of the Antigen During Myelopoiesis

By BRONISLAW PYTOWSKI,* THOMAS G. EASTON,‡ JAY E. VALINSKY,§ TINA CALDERON,‖ TSIEH SUN,¶ JUDITH K. CHRISTMAN,‖ SAMUEL D. WRIGHT,** AND JOSEF MICHL*

*From the *Department of Pathology and the ‡Department of Anatomy and Cell Biology, SUNY-Health Sciences Center at Brooklyn, Brooklyn, New York 11203; the §New York Blood Center Flow Cytometry Laboratory, New York, New York 10021; the ‖Department of Biochemistry, Mount Sinai School of Medicine, New York, New York 10029; the ¶Department of Pathology, North Shore University Hospital, Manhasset, New York 11030; and the **Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, New York 10021*

Cells of the myelomonocytic series, which arise from a single stem cell, express surface antigens that are either unique to granulocytes or monocytes or are shared with cells of other hematopoietic lineages (1–11). mAbs have been used as powerful means for the identification of these antigens. Some mAbs react with antigens present only during specific early stages of myelomonocytic differentiation, while others identify more differentiated lineage-specific plasma membrane antigens (2–4, 6, 7). mAbs have also been developed that identify proteins that constitute several leukocyte plasma membrane receptors for fragments of the third component of complement (8, 9). Since the same ligand is commonly recognized by more than one class of leukocytes, such antibodies typically identify all leukocytes that express the receptor for that particular ligand. For example, mAbs OKM1, OKM10, and IB4 identify the polypeptide complex comprising the human leukocyte receptor for C3bi (CR3) found on both neutrophils and monocytes (8, 9). However, recent reports have described the production of mAbs that immunoprecipitate proteins similar to those of the CR3 but that are found uniquely on human neutrophils (10, 11). These findings suggested the possibility that receptors whose ligand specificity is identical may exhibit antigenic and structural differences between the various classes of leukocytes.

In studies reported here, we set out to identify plasma membrane proteins of human neutrophils that carry antigenic determinants unique to this cell type. To increase the likelihood of producing mAbs to such neutrophil-specific antigens, we used a modification of a novel immunization procedure. Mice were first tolerized to common leukocyte antigens and then immunized with neutrophils.

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We describe the production of one mAb produced by this procedure and demonstrate that this mAb, called BH2-C6, is specific for human neutrophils. Combined analysis of human bone marrow cells and of the leukemic cell line HL-60 shows that the expression of the BH2-C6 antigen during hematopoietic differentiation is a property unique to cells committed to the neutrophil lineage. We further show that mAb BH2-C6 interferes with the CR3 activity of these cells, but the antigen recognized by this mAb is distinct from the neutrophil CR3.

Materials and Methods

Animals. Female BALB/c mice (5–10 wk old) were obtained from Charles River Breeding Laboratories (Wilmington, MA).

Preparation of Peripheral Blood Leukocytes. Polymorphonuclear (PMN)[1] and mononuclear (MN) leukocytes were isolated from heparinized (25 U/ml preservative-free sodium heparin) blood of healthy volunteers by dextran sedimentation followed by centrifugation in Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) (12). Residual erythrocytes were removed by hypotonic lysis with 0.2% sodium chloride. Lymphocytes were separated from monocytes by incubating the MN cells with acid-treated glass beads for 1 h at 37°C in 50% autologous serum. The nonadherent population contained <0.5% monocytes. Buffy coat cells were prepared as described above except the Ficoll-Hypaque step was omitted. Purified cells were stored in HBSS (Gibco Laboratories, Grand Island, NY) buffered to pH 7.2 with 10 mM Hepes (Sigma Chemical Co., St. Louis, MO). Viability remained above 97%, as judged by the trypan blue exclusion test.

Preparation of Bone Marrow Cells. Bone marrow was obtained from material removed from the head of the femur during hip replacement surgery. The cells were washed in HBSS containing 1 mM EDTA (Sigma Chemical, Co.), erythrocytes were removed by lysis for 5 min at room temperature (RT) in 0.15 M ammonium chloride in 10 mM $KHCO_3$, and the cells were resuspended in HBSS.

Cell Lines. P3U-1 myeloma cells, a gift of Dr. J. Unkeless of The Rockefeller University, were grown in DME (Gibco Laboratories) and 15% heat-inactivated FCS (HIFCS; Flow Laboratories, Inc., Rockville, MD) supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin (P/S; Flow Laboratories, Inc.). HL-60 cells (13), originally obtained from Dr. R. Gallo, National Institute of Health, Bethesda, MD, were grown in RPMI medium (Gibco Laboratories) and 10% HIFCS supplemented with P/S.

Induction of HL-60 Differentiation. HL-60 cells in log phase were induced to differentiate by 4 d of culture in 40 mM 5,5-dimethyl-oxazolidine-2,4-dione (DMO; Sigma Chemical Co.) (14) or 1.5 μM *cis*-retinoic acid (c-RA; a gift of Dr. Pestka, Hoffman-LaRoche Inc., Nutley, NJ) (15) or by 24-h culture in PMA (16). The degree of differentiation was quantitated using the nitro blue tetrazolium (NBT) reduction assay (17).

Preparation of the Hybridoma. Female BALB/c mice were injected intraperitoneally with 10^7 purified human lymphocytes. 1 d later they were injected intraperitoneally with 215 μg/g of body weight of Cytoxan (Mead Johnson Pharmaceutical, Evansville, IN) (18, 19). On days 13, 27, and 34, the mice were injected intraperitoneally with $1–2 \times 10^7$ purified human PMN. 3 d after the final boost, serum samples were obtained, and screened by indirect fluorescence assay (IFA) on human buffy coat cells for the presence of antibodies reactive with human PMN. The mouse chosen for cell fusion had the highest serum titer of anti-PMN antibodies and the lowest titer of antibodies to other human blood cells. Cell fusion was carried out according to Köhler and Milstein (20). After fusion, the cells were cultured in 96-well flat-bottomed plates (Falcon Labware, Oxnard, CA) in HAT medium (21). The cells were then transferred to hypoxanthine and thymidine

[1] *Abbreviations used in this paper:* c-RA, *cis*-retinoic acid; FITC-G anti-M, FITC-goat anti-mouse Ig; HIFCS, heat-inactivated FCS; IFA, indirect fluorescence assay; MN, mononuclear leukocyte; PMN, polymorphonuclear leukocyte; P/S, penicillin/streptomycin; SBTI, soybean trypsin inhibitor.

(HT) medium, and 20 d later supernatants were tested by IFA for antibodies reactive with PMN. Selected positive colonies were expanded and cloned in 0.55% soft agar (FMC Corp., Marine Colloids Division, Rockland, ME) in DME with 20% HIFCS and 13% Iscove's supplement and P/S using chick embryo fibroblasts as a feeder layer. Clone BH2.22.11 was selected because its supernatant selectively stained neutrophils in IFA. This clone was subcloned once, and subclone BH2-C6 was expanded for further use. Ascites was produced by injecting i.p. 10^7 hybridoma cells into pristane-primed (2,5,10,16-tetramethyl pentadecane; Sigma Chemical Co.) BALB/c mice.

Purification of BH2-C6. Globulin fraction of ascites was precipitated at 45% saturation of $(NH_4)_2SO_4$. Dialyzed precipitate was passed over a column of CM-Affi Gel Blue (Bio Rad Laboratories, Richmond, CA) equilibrated with PBS. BH2-C6 was contained in the flow-through peak.

Iodination of Antibodies. mAb BH2-C6 was iodinated by the chloramine T method (22) using carrier-free [^{125}I]Na (Amersham Corp., Arlington Heights, IL). The iodinated antibody was separated from free iodine by chromatography on coarse Sephadex G-25 (Pharmacia Fine Chemicals), dialyzed against PBS, and stored at −20°C. The specific activity of ^{125}I–BH2-C6 was 5–10 μCi/μg protein.

Radioimmunoassay (RIA). Purified PMN were seeded in 96-well flat-bottomed plates at 5×10^4 cells per well. The cells were fixed for 2 h in 1% formaldehyde in HBSS, washed, and blocked for 1 h with 1% HIFCS in HBSS (HBSS-HIFCS). The plates were placed on ice, and dilutions of ^{125}I–BH2-C6 antibody in HBSS-HIFCS were added in triplicate to the wells. For the determination of nonspecific binding, parallel triplicate wells received the same dilutions of ^{125}I–BH2-C6 containing 20 μg/well cold BH2-C6. After 1 h, the wells were washed, the cell-associated radioactivity was solubilized with 0.1% NP-40, and transferred to counting tubes with Q-tips. The data were analyzed using a one-sided, computer-generated, nonlinear regression.

Immunodiffusion. Immunodiffusion was performed using a commercial double immunodiffusion plate (CooperBiomedical, Inc., Malvern, PA).

Gel Electrophoresis. SDS-PAGE of Igs and cellular proteins was performed using standard methods (23). Purified cells were first treated with 1 mM diisopropylfluorophosphate (DFP; Sigma Chemical Co.) (24), washed in PBS, and lysed on ice in 1% NP-40 buffered with 10 mM Tris-HCl, pH 7.2, containing 0.9% NaCl, 0.25% sodium deoxycholate, 5 mM iodoacetamide, 1 mM PMSF, and 100 U/ml aprotonin (all from Sigma Chemical Co.). The nuclei were removed by centrifugation for 1 min at 8,000 *g*, the lysates were adjusted to 1% SDS and 1% 2-ME, boiled for 2 min, and the proteins were electrophoresed on 4–11% gradient gels.

Immunoblotting. Proteins were transferred electrophoretically from SDS-PAGE gels onto nitrocellulose as described elsewhere (25). A blot from one set of gel lanes was stained with 0.1% amido black. Duplicate blots were treated with 5% wt/vol nonfat dry milk (Carnation Co., Los Angeles, CA) dissolved in PBS (BLOTTO) (26). The blots were incubated overnight on a shaker at 5°C with ^{125}I–BH2-C6. Typically, 10^7 TCA-precipitable cpm were added in 80 ml BLOTTO. Blots were washed three times for 30 min in BLOTTO, rinsed in PBS, dried, and exposed to Kodak X-Omat x-ray film at −70°C.

Attachment of Opsonized Sheep E. E were opsonized with C3bi (EIgMC3bi) or IgG (EIgG) as described (27, 28). 5×10^4 purified human PMN in HBSS were allowed to adhere per 12-mm glass coverslip for 15 min at 37°C. The coverslips were placed on ice and the cells incubated for 30 min in 25 μl HBSS containing 2.5 μg of BSA or mAbs in the presence or absence of 2 mg/ml soybean trypsin inhibitor (SBTI, Sigma Chemical Co.). 2.5×10^6 of EIgMC3bi or EIgG were added in 25 μl HBSS to each coverslip. After incubation for 1 h at 37°C, nonbound E were removed by dipping the coverslips into HBSS, and the cells were fixed in 2% glutaraldehyde in HBSS. The binding was enumerated by counting cells in several fields with a × 100 objective in a phase-contrast microscope.

Indirect Fluorescence Assay (IFA). Hybridoma supernatant screening was done on buffy coat cells or purified PMN adhered to Terasaki plates (Falcon Labware). All other assays were done with cells seeded onto 12-mm glass coverslips (Bellco Glass, Inc., Vineland,

NJ). For IFA on buffy coat and HL-60 cells, the coverslips were pretreated for 30 min at RT with 50 μg/ml poly-L-lysine (PLL; Sigma Chemical Co.). The cells were incubated with hybridoma culture supernatants or dilutions of primary antibody in HBSS-HIFCS for 45 min at 2°C. Nonbound antibodies were washed off by dipping the coverslips into ice-cold PBS-HIFCS. The cells were fixed in freshly prepared 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in PBS. Affinity-purified rhodaminated goat anti-mouse IgM (TRITC-G anti-M IgM, μ chain-specific; CooperBiomedical, Inc.) second antibody was added for 30 min on ice. The cells were photographed on Tri-X film (Kodak Inc., Rochester, NY) in a Zeiss Standard microscope equipped with phase contrast and epifluorescence optics.

Flow Cytometry. Cells were incubated for 1 h on ice with 1 μg/ml BH2-C6 in HBSS-HIFBS, washed by centrifugation in HBSS-HIFCS, and incubated for 30 min on ice with a 1:40 dilution of affinity-purified IgG fraction of goat anti-mouse Ig conjugated with FITC (FITC-G anti-M Ig; CooperBiomedical, Inc.). The cells were washed free of unbound antibody, and resuspended in ice-cold PBS-HIFCS. Fluorescence was analyzed in a Becton Dickinson & Co. (Mountain View, CA) FACS IV flow cytometer equipped with a Spectra-Physics 2-W argon-laser. Excitation was at 488.8 nm at an incident power of 300 mW. Fluorescence emission was measured using a 530 nm long-bandpass filter and recorded as the log of relative intensity. Data were analyzed using a Becton Dickinson & Co. Consort 30 computer system. Sorted cells were centrifuged onto glass slides using a cytocentrifuge (Shandon-Eliot, Inc., Camberlay, England), fixed in methanol, stained with Wright-Giemsa stain, and photographed.

Immunoelectron Microscopy. Human PMN were incubated in suspension on ice for 1 h with 20 μg/ml BH2-C6 in PBS-HIFBS, washed by centrifugation in HBSS-HIFCS, and sequentially incubated on ice with a rabbit anti-mouse Ig (Miles Laboratories Inc., Elkhart, IN) diluted 1:20 in HBSS-HIFCS and a 1:10 dilution of 9 nm colloidal gold prepared by the method of Mühlpfordt (29) and derivatized with staphylococcal protein A. After washing, cell pellets were fixed in 2% glutaraldehyde in PBS, postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Epon. Thin sections were stained in uranyl acetate, and examined on a Phillips 300 TEM.

Results

Preparation and Characterization of the BH2-C6 Antibody. mAb BH2-C6 is an IgM with κ light chains, as demonstrated by Ouchterlony immunodiffusion with rabbit antisera specific for mouse μ and κ chains. Upon complete reduction and analysis by SDS-PAGE, the molecule separates into heavy and light chains of 80 and 27 kD, respectively (data not shown).

Specificity of the mAb BH2-C6 for Human Peripheral Blood Neutrophils. The reactivity of BH2-C6 with human cells was examined by IFA. Fig. 1 shows the resulting fluorescence pattern on living human leukocytes stained with 5 μg/ml BH2-C6. Purified neutrophils (Fig. 1 *c*) stain in a bright, uniform circular pattern (Fig. 1 *d*). This staining with BH2-C6 is characteristic of >99% of unspread neutrophils. When buffy coat cells were examined, only the neutrophils were found to stain with BH2-C6 (Fig. 1 *f*). Unstained cells visible in the phase-contrast micrograph of Fig. 1 *e* include lymphocytes (*arrowheads*), monocytes (*large arrows*), and platelets (*small arrow*). In the absence of BH2-C6, there is no fluorescent staining (Fig. 1 *b*) of purified PMN (Fig. 1 *a*). Further, BH2-C6 failed to stain human erythrocytes, foreskin fibroblasts, as well as frozen sections of lymph nodes. mAb BH2-C6 does not react with neutrophils of mice, rats, and macaque monkeys (data not shown).

BH2-C6 is cytotoxic. Incubation of BH2-C6–coated neutrophils with fresh guinea pig complement at 37°C results in the lysis of >99% of the cells.

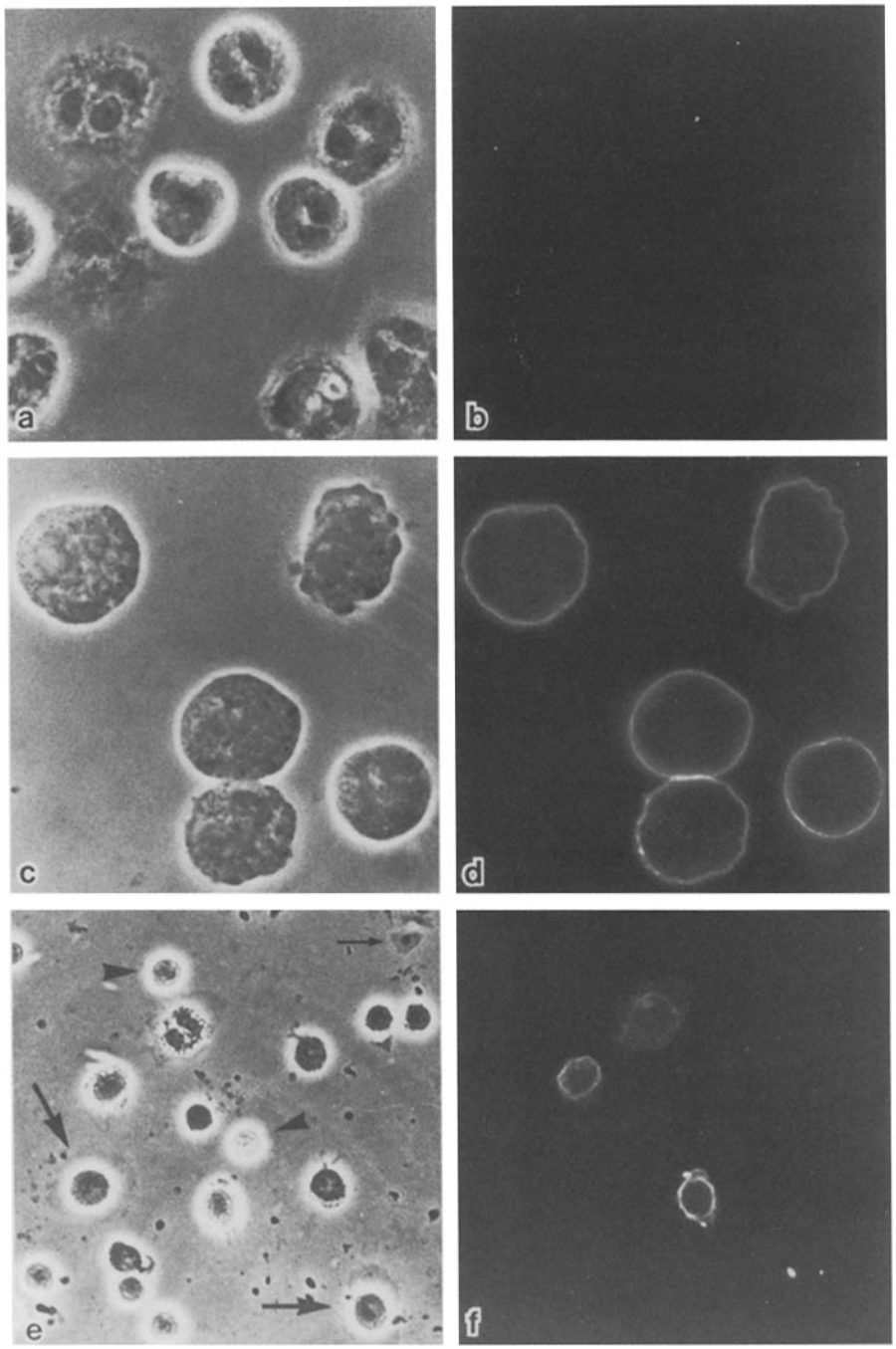

FIGURE 1. Indirect fluorescence of human peripheral cells stained with mAb BH2-C6. PMN (*a–d*) and buffy coat leukocytes (*e* and *f*) were purified, allowed to adhere to PLL-coated coverslips, and processed as described in the Materials and Methods section. No staining of the PMN can be seen in the absence of mAb BH2-C6 (*b*). Addition of the mAb BH2-C6 results in the appearance of ring fluorescence of the neutrophils (*d*). Treatment of the buffy coat cells with mAb BH2-C6 results in fluorescence staining limited to neutrophils (*f*). There is no fluorescence on the plasma membranes of monocytes (*large arrow*), lymphocytes (*arrowheads*), and platelets (*small arrow*) that can be identified in the phase-contrast photomicrograph shown in *e*. The photomicrographs were taken with a × 100 (*a–d*) and × 40 (*e* and *f*) objectives. (*a*, *c*, and *e*) Phase-contrast; (*b*, *d*, and *f*) corresponding epifluorescence images.

Lack of Reactivity of BH2-C6 with Human Eosinophils. In IFA analysis, we consistently observed that mAb BH2-C6 does not stain eosinophils in the ring pattern characteristic of plasma membrane antigens. However, the presence of an autofluorescent substance in the granules of eosinophils made it difficult to determine, on the basis of IFA, whether these cells express the surface antigen recognized by mAb BH2-C6. To further investigate the specificity of mAb BH2-C6 for human neutrophils, we used immunogold electron microscopy. Purified PMN in ice-cold HBSS-HIFCS were sequentially reacted with mAb BH2-C6, rabbit anti-mouse Ig, and colloidal gold derivatized with protein A. Fig. 2, *b* and *c*, shows binding of the gold particles around the entire periphery of the neutrophil. By contrast, the plasma membrane of the eosinophil (Fig. 2*c*), identified by the presence of the crystalloids in the granules (*arrowheads*), has no bound gold particles. Of five eosinophils observed in thin sections, none stained with the BH2-C6–containing gold immunocomplex, while virtually all neutrophils observed were stained.

When observed at higher magnification (Fig. 2*d*), the linear deposition of gold on the surface of the neutrophil indicates that the BH2-C6 antigen is uniformly distributed in the plasma membrane of this cell with no apparent clustering or patching. The high density of the deposited gold suggests the presence of a large number of BH2-C6 antigenic sites on the neutrophil plasma membrane. In the absence of BH2-C6, the plasma membrane of neutrophils is completely devoid of the gold particles (Fig. 2*a*).

Identification of the Neutrophil Molecule Recognized by BH2-C6. The biochemical nature of the molecule recognized by BH2-C6 was investigated with immunoblotting of PMN proteins separated by SDS-PAGE under reducing conditions. Separated proteins from lysates of 10^7 PMN and mononuclear cells were transferred electrophoretically onto nitrocellulose, and the band containing the epitope recognized by BH2-C6 was detected with ^{125}I-labeled BH2-C6. Fig. 3, lane *4*, shows that ^{125}I–BH2-C6 binds to a PMN polypeptide band with an average molecular mass of 157 ± 12 kD (BH2-Ag). The specificity of this binding was shown by the inability of ^{125}I–BH2-C6 to bind to PMN proteins in the presence of 50-fold excess of unlabeled antibody (lane *5*). The absence of binding of ^{125}I–BH2-C6 to proteins of mononuclear cells (lane *3*) confirms that the BH2-Ag is not present in these cells.

Quantitation of Binding of ^{125}I–BH2-C6 to Human PMN. Binding of ^{125}I–BH2-C6 to human PMN is saturable, with a half-saturating concentration of ~325 ng antibody per 5×10^4 (Fig. 4). Computer-generated nonlinear fit of the data gives an estimate of 19.5 ng of antibody bound to this number of cells at saturation, corresponding to 214,000 molecules of ^{125}I–BH2-C6 bound per cell.

Effect of mAb BH2-C6 on the Neutrophil Receptor for C3bi (CR3). mAbs reported to be neutrophil specific (10–11) have been shown to immunoprecipitate proteins similar to those of the CD11/CD18 complex of leukocyte antigens. These antigens comprise a family of three dimeric proteins, CD11a/CD18 (also known as LFA-1), CD11b/CD18 (also known as Mac-1 or the C3bi receptor [CR3]), and CD11c/CD18 (also known as p150,95) (8, 9). Since the BH2-Ag has a molecular weight closely resembling that of CD11b and CD11c, we tested the ability of the

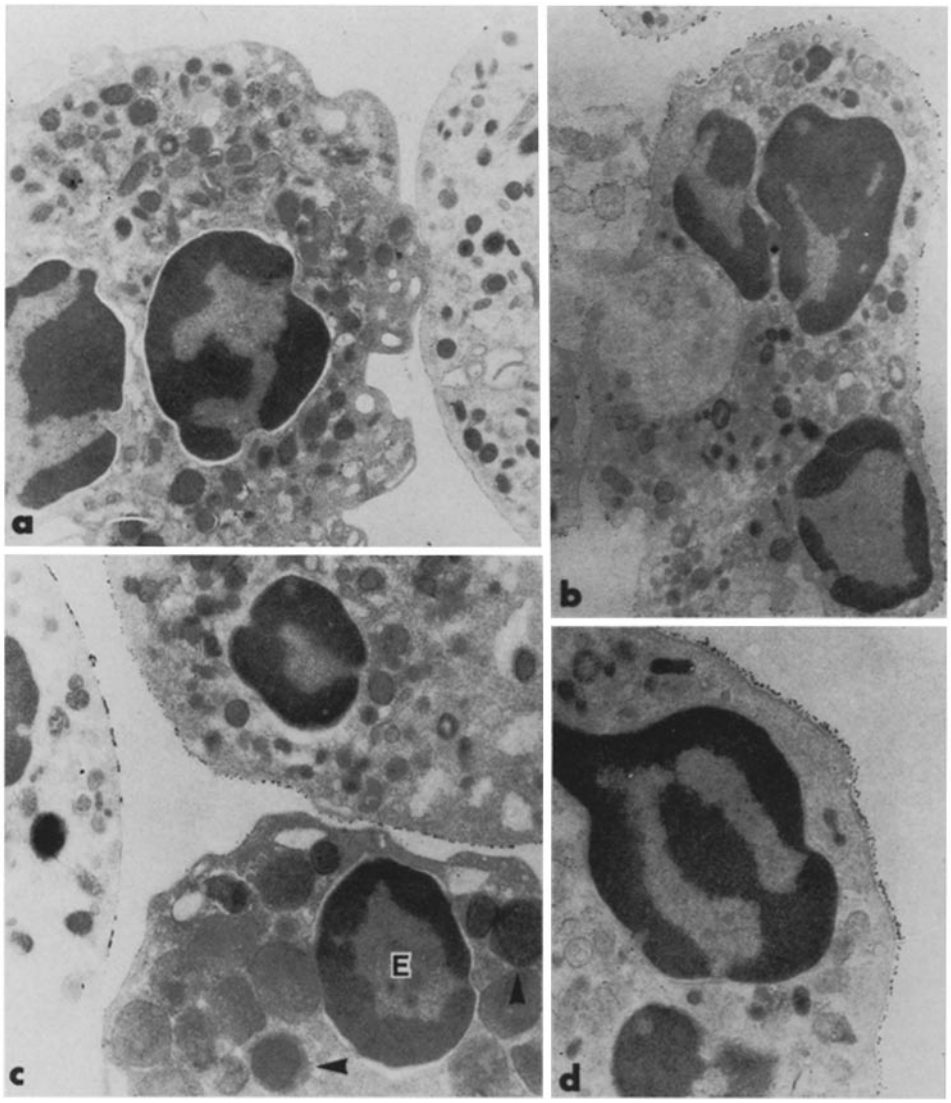

FIGURE 2. Immunoelectron microscopy of human PMN stained with mAb BH2-C6. Purified PMN were sequentially stained with mAb BH2-C6, rabbit anti-mouse Ig, and protein A–derivatized 9 nM colloidal gold and prepared for electron microscopy as described in Materials and Methods. Control samples were processed in the absence of mAb BH2-C6. (*a*) Control cells. (*b–d*) Samples processed in the presence of mAb BH2-C6. The arrowheads in *c* indicate the characteristic crystalloids found in the granules of eosinophils (*E*). Magnifications: *a*, × 14,200; *b*, × 12,600; *c*, × 14,600; *d*, × 19,400.

mAb BH2-C6 to interfere with binding of sheep erythrocytes opsonized with C3bi to PMN.

Efficient binding of C3bi-opsonized E to phagocytes occurs only at temperatures above 30°C (30). It is possible that during incubation at this temperature, the release of endogenous proteases by the neutrophils may lead to loss of the

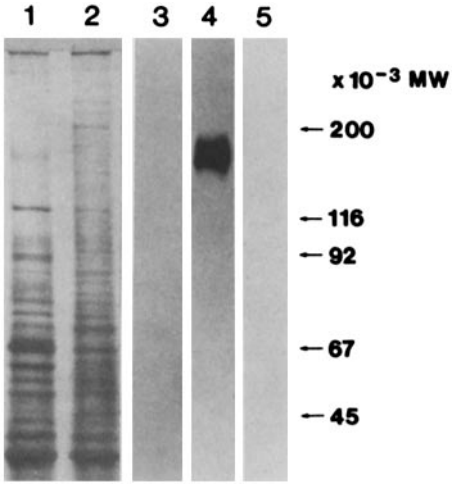

FIGURE 3. Identification of the antigen recognized by the mAb BH2-C6. Solubilized proteins of PMN and mononuclear cells were separated by SDS-PAGE on 4–12% gels using 10^7 cells per lane. The separated proteins were blotted onto nitrocellulose as described in Materials and Methods. Parallel blots were stained with amido black (*lanes 1* and *2*) or incubated with ^{125}I–BH2-C6 and autoradiographed (lanes *3–5*). (Lanes *1* and *3*) Mononuclear cells. (Lanes *2*, *4*, and *5*) PMN. (Lane *5*) PMN blots reacted with ^{125}I–BH2-C6 in the presence of 50-fold excess of unlabeled BH2-C6.

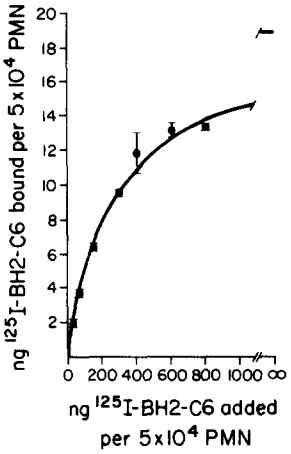

FIGURE 4. Binding characteristics of ^{125}I-BH2-C6. Purified PMN were seeded at 5×10^4 per well in 96-well plates and incubated with increasing amounts of ^{125}I–BH2-C6. Nonspecific binding was determined by incubating the cells with ^{125}I–BH2-C6 in the presence of 20 μg/well of unlabeled mAb BH2-C6. Computer-generated nonlinear fit of specific binding of ^{125}I–BH2-C6 to PMN gives an estimate of 19.5 ng of ^{125}I–BH2-C6 bound to 5×10^4 cells at saturation.

C3bi ligand or the CR3. Therefore, the binding experiments were done in the absence or presence of the serine protease inhibitor SBTI.

The addition of mAb BH2-C6 reduced the attachment index for EIgMC3bi to 2% of the BSA control, but this effect was partially reversed (to 41% of control) by the addition of SBTI (Table I). By contrast, the addition of the mAb BH2-C6 had no effect on the ability of the PMN to bind EIgG (Table I). Control experiments showed that the binding of EIgG was completely inhibited by the presence of the anti-FcR mAb 3G8 (31), and this inhibition was not reversed by SBTI.

Three possibilities are suggested by these observations: (*a*) BH2-C6 may bind to CR3; (*b*) BH2-C6 may bind to a molecule physically associated with CR3 and thus sterically block ligand binding; (*c*) BH2-C6 may trigger the release of proteolytic enzymes that cleave C3bi or its receptor. The following data rule out the first two possibilities.

BH2-C6 Does Not Recognize Antigens in the CD18 Complex. We examined the expression of BH2-C6 Ag on PMN from a patient congenitally deficient in all

TABLE I

The Effect of mAb BH2-C6 on the Attachment of Opsonized Sheep Erythrocytes by Human Polymorphonuclear Leukocytes

Treatment*	Test particles‡							
	EIgMC3bi				EIgG			
	−SBTI		+SBTI§		−SBTI		+SBTI	
		%		%		%		%
BSA	848	100	926	100	469	100	501	100
BH2-C6	20	2	379	41	502	107	441	88
3G8	642	76	1014	109	2	<1	4	<1

* BSA and mAbs BH2-C6 and 3G8 at 100 μg/ml.

‡ Attachment index is defined as: [(Number of E attached)/(total number of PMN)] × (percent of PMN with attached E). These data are from a representative experiment; two additional experiments gave similar results.

§ SBTI at 2 mg/ml.

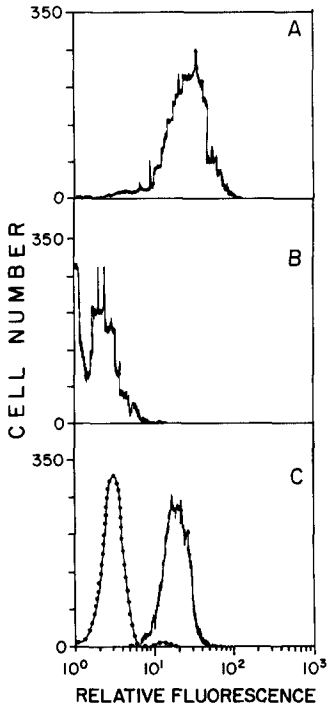

FIGURE 5. Cytofluorimetric analysis of mAb BH2-C6 reactivity with neutrophils of a patient deficient in the CD18 complex. Purified PMN from a patient with congenital absence of CD18 and from an age-matched normal subject were incubated with 5 μg/ml mAb BH2-C6 or the anti-CD18 mAb IB4 followed by FITC–G anti–M Ig. (*A*) Fluorescence distribution of purified PMN from the normal subject stained with mAb IB4. (*B*) The pattern of fluorescence obtained by staining PMN from CD18-deficient patient with mAb IB4. (*C*) Fluorescence pattern obtained by staining the patient's PMN with mAb BH2-C6. Dotted line shows the fluorescence distribution of the patient's PMN when mAb BH2-C6 was omitted.

three members of the CD18 complex (Wright, S. D., Z. Chad, and L. Kabbash, manuscript in preparation). mAb IB4 (directed against CD18, reference 8) stains normal PMN brightly (Fig. 5*a*), but no staining above background is detected on PMN from the patient (Fig. 5*b*). In contrast, the PMN from the patient react normally with BH2-C6 (Fig. 5*c*). These data were confirmed by measuring the binding of radioiodinated IB4 and BH2-C6 to purified PMN. Binding of IB4 was <0.2% of that on control cells, while binding of BH2-C6 was 80% as great as control (data not shown). These data demonstrate that BH2-C6 does not

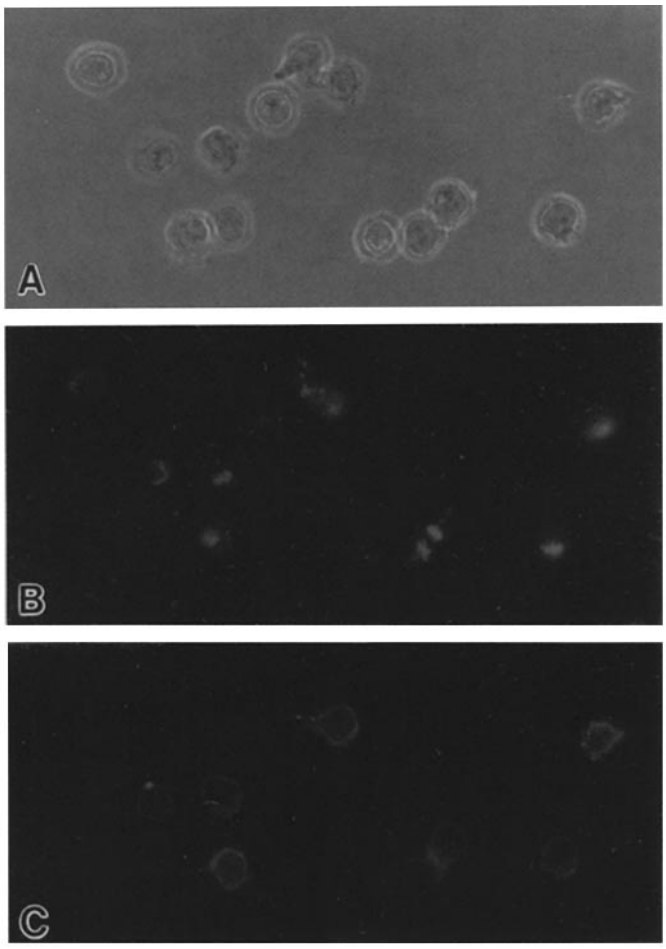

FIGURE 6. Lack of cocapping of the BH2-Ag and the CR3. Purified PMN were sequentially incubated in suspension on ice with 20 μg/ml mAb OKM1 and FITC–G anti–M IgG and washed by centrifugation in ice-cold PBS. The cells were resuspended in 1 ml of HBSS at 37°C for 5 min to allow capping, cooled to 5°C with 40 ml of ice-cold PBS and allowed to adhere on ice to 12 mm coverslips coated with PLL. The cells were then fixed in 4% paraformaldehyde and further sequentially incubated on ice with 5 μg/ml mAb BH2-C6 and TRITC–G anti–M IgM. Photomicrographs were taken with a × 63 objective. (*A*) Phase-contrast; (*B*) mAb OKM1; (*C*) mAb BH2-C6.

recognize any members of the CD18 family and thus does not recognize the CR3.

BH2-C6 Ag Does Not Cocap with CR3. Purified human PMN were sequentially incubated on ice in suspension with the anti-CR3 mAb OKM1 (9) and FITC–G anti–M IgG. The cells were warmed to 37°C for 5 min to allow the capping of the OKM1-CR3 complex, cooled to 5°C, and adhered to PLL-coated coverslips at 5°C. The cells were fixed in paraformaldehyde, incubated sequentially with mAb BH2-C6 and TRITC–G anti–M IgM (μ chain–specific), and photographed. As can be seen in Fig. 6, the BH2-Ag does not segregate into the OKM1-

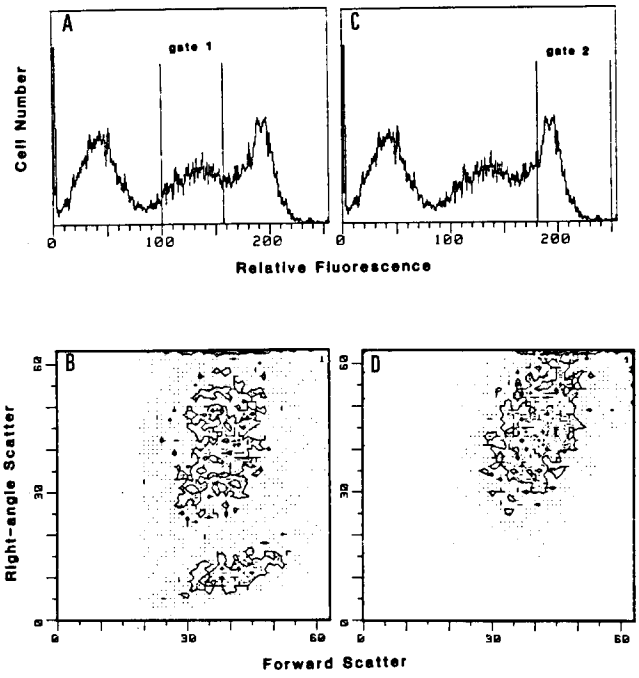

FIGURE 7. FACS analysis of mAb BH2-C6 binding to human bone marrow cells. Bone marrow cells were serially incubated with mAb BH2-C6 and FITC–G anti–M Ig and sorted as described in Materials and Methods. (*A* and *B*) Distribution of fluorescence intensity and settings of the gates for sorting and analysis. (*C* and *D*) Forward and right angle light scatter plots of gated fluorescence-positive cell populations.

mediated surface caps. This result demonstrates that the BH2-Ag is not physically associated with the CR3 in the plasma membrane.

Expression of BH2-Ag by Myelomonocytic Precursor Cells in Normal Human Bone Marrow. The specificity of mAb BH2-C6 for a plasma membrane antigen unique to human peripheral blood neutrophils prompted us to investigate the expression of this antigen during myelopoiesis. Cytofluorometric analysis of human bone marrow cells was used in conjunction with histochemical staining and differential counting of sorted cells to establish the distribution of BH2-Ag during neutrophilic differentiation.

5×10^6 human bone marrow cells were stained for 1 h on ice with 1 μg/ml mAb BH2-C6 and FITC–G anti–M Ig. In all samples examined, the cellular distribution based on light scatter and differential cell counts was characteristic of normal bone marrow. Analysis of fluorescence shows a cell population negative for BH2-C6–mediated fluorescence and two populations of intermediate and high fluorescence intensity (Fig. 7, *A* and *C*). Together, the positive populations contained 65% of the bone marrow cells analyzed. The cells making up these two populations were sorted using fluorescence intensity gating shown in Fig. 7, *A* and *C*. Differential counts were performed on cytocentrifuge preparations of the sorted cells after Wright-Giemsa staining (Table II). The cells in the brightly fluorescent population (Fig. 7*C*, gate 2) have light scatter characteristics of late-stage granulocytic precursors and of mature granulocytes (Fig. 7*D*). The population of cells characterized by intermediate-intensity fluorescence (Fig. 7*A*, gate 1) has a light-scatter distribution (Fig. 7*B*) that partially overlaps that of the high-intensity population. However, the bone marrow cells gated in panel *A* include,

TABLE II

Cytofluorimetric Analysis of Normal Human Bone Marrow Cells Based on Reactivity with mAb BH2-C6

Cells	Unsorted	Distribution of cells sorted according to fluorescence		
		Negative	Low intensity	High intensity
	%	%	%	%
Myeloblasts	1	7	0	0
Promyelocytes	6	2	26	0
Myelocytes	19	0	55	15
Metamyelocytes	16	0	10	28
Bands	14	0	1	22
Neutrophils	14	0	0	35
Eosinophils	3	2	6	0
Basophils	1	0	0	0
Mononuclear cells*	16	76	2	2
Erythroid cells‡	9	12	0	0
Others§	2	1	0	0

* Monocytes and lymphocytes.
‡ Pronormoblasts, basophilic normoblasts, polychromatic normoblasts and orthochromatic normoblasts.
§ Plasmacytes and megakaryocytes.

in addition, a subpopulation whose light-scatter distribution (Fig. 7*B*) is characteristic of cells in early stages of myelopoiesis (32).

All myeloblasts observed were negative for BH2-Ag. The earliest granulocytic precursors found to be fluorescence-positive for BH2-Ag are the promyelocytes (Table II). As the cells mature into myelocytes, there is a continued, low level expression of BH2-Ag. During this stage, there occurs a significant increase in BH2-Ag surface expression as evidenced by the division of myelocytes into the intermediate and high fluorescence intensity populations. The high fluorescence intensity population is composed of metamyelocytes, banded, and segmented neutrophils.

Expression of BH2-Ag on HL-60 Cells During Neutrophilic Differentiation. In an attempt to confirm in vitro the results of bone marrow analysis, we examined the expression of BH2-Ag on the human myeloblastic–promyelocytic cell line HL-60 (13). This cell line can be induced to further differentiate along the granulocytic lineage by treatment with various pharmacological agents. In the first experiment, we examined the binding of ^{125}I–BH2-C6 to HL-60 cells cultured in the absence or presence of 40 mM DMO (14) or 1.5 μM c-RA (15). After an incubation period of 4 d, the cells were seeded in 96-well, flat-bottomed plates pretreated with 50 μg/ml PLL to promote equal adherence of control and induced cells. A saturating dose of 37 ng ^{125}I–BH2-C6 and 250 ng cold BH2-C6 (see Fig. 4) was added to each well for 1 h on ice and the cells were processed as described in Materials and Methods. Table III shows the results of this experiment. HL-60 cells that were cultured in RPMI alone bind only 2.3% of the amount of ^{125}I–BH2-C6 bound by an equivalent number of PMN. Incubation of

TABLE III
Effect of Neutrophilic Differentiation on the Binding of ^{125}I-BH2-C6 to HL-60 Cells

Cells*	Treatment of cells	Specific cpm bound‡	Cells reducing NBT
			%
PMN	0	15,960 ± 1,055§	ND
HL-60	0	368 ± 143	8
HL-60	1.5 μM c-RA	3,136 ± 51	27
HL-60	40 mM DMO	3,926 ± 204	41

* 5×10^4 cells per well.
‡ Specific cpm were calculated by subtracting the cpm bound in the presence of 50-fold excess of unlabeled mAb BH2-C6.
§ Standard error of the mean.

the cells with c-RA results in an 8.5 times higher binding of ^{125}I–BH2-C6. Similarly, incubation with DMO increases the binding of ^{125}I–BH2-C6 by 10.7 times.

Parallel cell samples were evaluated for NBT reduction. The results in Table III show that only 8% of HL-60 cells cultured in RPMI alone are capable of reducing NBT. By contrast, when HL-60 cells are incubated in the presence of c-RA, the percentage of cells reducing NBT increases 3.4-fold. The corresponding increase for cells incubated with DMO is 5.1-fold.

The level of ^{125}I–BH2-C6 binding to HL-60 cells induced with c-RA or DMO is only 20 and 25%, respectively, of that binding to PMN (Table III). Thus, it was important to establish whether the incubation of HL-60 cells with these agents results in the induction of BH2-Ag expression in a subpopulation of these cells. To address this question, we used indirect immunofluorescence to determine the percentage of BH2-Ag$^+$ HL-60 cells. As shown in Fig. 8, only very few (<4%) BH2-Ag$^+$ cells are found in an HL-60 cell population that was incubated in RPMI alone (Fig. 8*B*). By contrast, 82% of cells induced with DMO (Fig. 8*D*) and 90% of cells induced with c-RA (Fig. 8*F*) express the BH2-Ag.

These results show that the treatment with inducers of granulocytic differentiation, DMO or c-RA, stimulates the HL-60 cells to initiate expression of BH2-Ag. The appearance of this antigen correlates well with cellular changes which indicate granulocytic differentiation: alteration of cell morphology (Fig. 8, *A*, *C*, and *E*) and the ability to reduce NBT. In contrast, the exposure of HL-60 cells to TPA, an inducer of monocytic differentiation, failed to evoke the appearance of BH2-Ag (data not shown).

Discussion

This report describes an mAb that exhibits specificity for human peripheral blood neutrophils and for several stages of differentiation of these cells. Analysis of cells by immunofluorescence (Fig. 1) clearly shows that the reactivity of mAb BH2-C6 is restricted to PMN. In numerous assays with blood of several donors, BH2-C6 staining of monocytes, lymphocytes, erythrocytes, or platelets was never observed. Furthermore, in analysis by immunoelectron microscopy, we found no

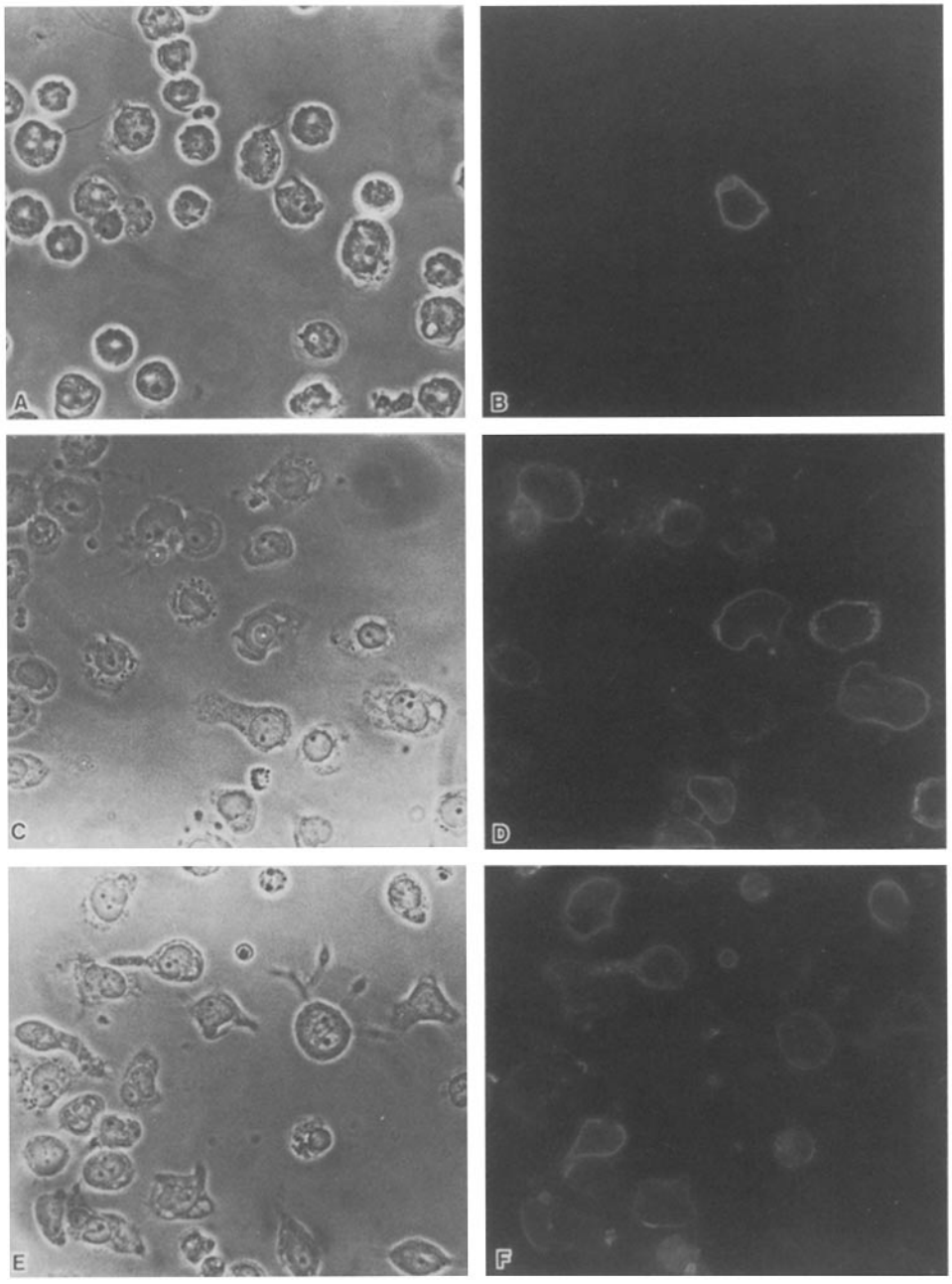

FIGURE 8. Expression of BH2-Ag by HL-60 cells. HL-60 cells were grown for 4 d before analysis in culture medium alone (*A* and *B*) and in medium containing DMO (*C* and *D*) or c-RA (*E* and *F*). For analysis, the cells were seeded on PLL-coated glass coverslips, sequentially incubated with mAb BH2-C6 and TRITC–G anti–M IgM, fixed and photographed using a × 63 objective. (*A*, *C*, and *E*) Phase contrast. Note the increase in cell spreading and the appearance of dense cytoplasmic granules in *C* and *E*. (*B*, *D*, and *F*) Corresponding fluorescence.

BH2-C6–mediated staining of eosinophil plasma membranes under conditions in which all the neutrophils observed showed surface staining (Fig. 2). The uniform distribution of the gold-containing immune complexes on the plasma membrane of the neutrophils (Fig. 2) correlates well with the uniform pattern of fluorescence that is characteristic of BH2-C6 staining. This finding demonstrates that BH2-Ag is not located in membrane clusters such as those reported for the PMN type 1 complement receptor (33).

Analysis by immunoblotting of total cellular proteins separated from PMN and mononuclear cells under reducing conditions shows that the molecule recognized by mAb BH2-C6 is a single protein of 157 kD. The width of the band seen on autoradiographs (157 ± 12 kD) suggests that BH2-Ag may be heterogeneously glycosylated (Fig. 3). The lack of binding of ^{125}I–BH2-C6 to proteins of mononuclear cell lysates indicates that monocytes and lymphocytes do not possess intracellular BH2-Ag or other proteins that carry the BH2-C6-specific determinant.

Binding of mAb BH2-C6 to neutrophils significantly reduces attachment of C3bi-coated erythrocytes to these phagocytes, thus suggesting an association of the BH2-C6 Ag with CR3. We find, however, that the Ag recognized by BH2-C6 is *not* expressed on CR3 (CD11b/CD18), nor on the structurally related CD11a/CD18 or CD11c/CD18 molecules. Cells from a patient deficient in all three members of the CD18 complex react well with BH2-C6 (Fig. 5). We further observed that the BH2-C6 Ag does not cocap with CR3 (Fig. 6), indicating that it is not associated with CR3 in the plasma membrane.

An explanation for the effects of BH2-C6 on CR3 function is suggested by the observation that its inhibitory effects are suppressed by high concentrations of protease inhibitor (Table I). Thus, BH2-C6 may trigger the release of proteases that cleave C3bi or its receptor. Two of the neutrophil serine proteases, elastase and cathepsin G (37), have been shown by others to convert C3bi to C3d, which is not recognized by CR3 (34). Stimulation of secretion of endogenous proteases by antibodies to cell surface proteins has also been reported (35, 36). While the function of BH2-C6 Ag is still obscure, our data suggest that this antigen may function to trigger secretion by PMN. Studies to examine this possibility are currently under way.

Several mAbs have been described in recent years that appear to be specific for human granulocytes (10, 11, 39–41). Frequently, these mAbs recognize sugar sequences on glycoproteins and glycolipids (10, 39). One highly antigenic oligosaccharide, lactose-*N*-fucopentose III (5) is the epitope of the mAb PMN-7C3 (10) and the AHN series of human granulocyte-specific mAbs (40). Although the exact nature of the epitope recognized by mAb BH2-C6 remains to be defined, we believe that mAb BH2-C6 recognizes a structure distinct from this oligosaccharide. This conclusion is based on the absence of BH2-C6 reactivity with uninduced HL-60 cells and human myeloblasts, both of which express this oligosaccharide antigen (39, 42). Furthermore, mAb BH2-C6 recognizes a single neutrophil polypeptide, while mAbs AHN 1–4 identify two proteins with identical antigenic determinants (40).

The BH2-Ag molecule is expressed on the plasma membrane of all the cells committed to neutrophilic differentiation starting with the promyelocyte. In all

bone marrow samples analyzed by cytofluorimetry, the myeloblasts were identified only in the fluorescence-negative population. This correlates well with the finding that the human cell line HL-60 is also negative for BH2-Ag. These cells are arrested in the myeloblastic–promyelocytic stage of development but can be induced to further differentiate along granulocytic or monocytic lineages (13, 17). Upon induction with c-RA or DMO, 60–90% of HL-60 cells attain the morphological, enzymatic, and functional characteristics of myelocytes (15–16 and this paper). Based on our finding that the myelocytes in cytofluorimetric analysis fall predominantly into the fluorescent fraction of intermediate intensity, it could be expected that c-RA or DMO-induced HL-60 cells would express relatively low levels of BH2-Ag as compared with mature neutrophils. In fact, exactly this finding was obtained as shown by RIA (Table III) and IFA (Fig. 8). It should be noted that the percentage of DMO and c-RA-induced HL-60 cells that express BH2-Ag is significantly greater than the percentage of these cells capable of reducing NBT. This suggests that the expression of BH2-Ag is an earlier indicator of the granulocytic differentiation of these cells than the ability to generate the superoxide anion.

During the myelocytic stage, there is a significant increase in the plasma membrane expression of BH2-Ag. This is shown by the appearance of myelocytes in the high fluorescence intensity fraction of bone marrow cells sorted by cytofluorimetry. This increase, therefore, occurs at the time when the mitotic divisions cease and the cells initiate maturation into segmented neutrophils. At this stage of neutrophilic development, there is also a significant increase in the expression of the plasma membrane receptors for complement (38), Fc (38), and chemotactic peptides (43), all of which are critical for the function of mature neutrophils. The role played by the BH2-Ag in the biology of the human neutrophil is currently being investigated.

Summary

We have used mice selectively tolerized to antigens of human lymphocytes by treatment with cyclophosphamide to raise an mAb, BH2-C6, that reacts with a plasma membrane antigen specific for human neutrophils. This specificity is demonstrated by indirect immunofluorescence microscopy, cytochemical analysis of fluorescence-positive and -negative cell populations separated by flow cytometry, and by the selective, complement-mediated killing of mAb BH2-C6–treated neutrophils. Additional evidence for the neutrophil specificity of mAb BH2-C6 is shown by immunoelectron microscopy, which demonstrates a lack of reactivity with human eosinophils. Immunoblotting of SDS-PAGE–separated proteins of polymorphonuclear leukocytes with ^{125}I-labeled BH2-C6 identifies protein with an average molecular mass of 157 kD. Binding studies show that, at saturation, neutrophils bind 214,000 molecules of ^{125}I–BH2-C6 per cell.

Addition of mAb BH2-C6 to neutrophils significantly reduces the number of C3bi-opsonized sheep erythrocytes (EIgMC3bi) bound by these cells. This reduction is partly reversed by the presence of soybean trypsin inhibitor (SBTI), indicating that at least one part of this inhibition is due to BH2-C6–stimulated secretion of a serine protease that may affect ligand binding.

Cytochemical analysis of normal human bone marrow cells sorted by cytofluo-

rimetry identifies the promyelocyte as the precursor cell that first expresses BH2-Ag on the plasma membrane. Using the leukemic cell line HL-60, we demonstrate that only inducers of granulocytic differentiation, *cis*-retinoic acid, and dimethyloxazolidine stimulate the expression of BH2-Ag. These results show that the expression of BH2-Ag during myelomonocytic differentiation is a property uniquely possessed by cells committed to the neutrophilic lineage.

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