CD164, a Novel Sialomucin on CD34+ and Erythroid Subsets, Is Located on Human Chromosome 6q21

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CD164 is a novel 80- to 90-kD mucin-like molecule expressed by human CD34+ hematopoietic progenitor cells. Our previous results suggest that this receptor may play a key role in hematopoiesis by facilitating the adhesion of CD34+ cells to bone marrow stroma and by negatively regulating CD34+ hematopoietic progenitor cell growth. These functional effects are mediated by at least two spatially distinct epitopes, defined by the monoclonal antibodies (MoAbs) 103B2/9E10 and 105A5. In this report, we show that these MoAbs, together with two other CD164 MoAbs, N6B6 and 67D2, show distinct patterns of reactivity when analyzed on hematopoietic cells from normal human bone marrow, umbilical cord blood, and peripheral blood. Flow cytometric analyses revealed that, on average, 63% to 82% of human bone marrow and 55% to 93% of cord blood CD34+ cells are CD164+, with expression of the 105A5 epitope being more variable than that of the other identified epitopes. Extensive multiparameter flow cytometric analyses were performed on cells expressing the 103B2/9E10 functional epitope. These analyses showed that the majority (>90%) of CD34+ human bone marrow and cord blood cells that were CD38lo or that coexpressed AC133, CD90(Thy-1), CD117(c-kit), or CD135(FLT-3) were CD164(103B2/9E10)+. This CD164 epitope was generally detected on a significant proportion of CD34+CD71(bright) or CD34+CD33(dim) cells. In accord with our previous in vitro progenitor assay data, these phenotypes suggest that the CD164+CD34+ and CD164+CD33+ cells in bone marrow are mainly CD19− B-cell precursors, with the CD164+CD33+ and CD34+CD20− B cells in bone marrow, but being virtually absent from B cells in the peripheral blood. Further analyses of the CD34+CD164+CD33+ subsets indicated that one of the most prominent populations consists of maturing erythroid cells. The expression of the CD164+CD33+ subset precedes the appearance of the glycophorin C, glycophorin A, and band III erythroid lineage markers but is lost on terminal differentiation of the erythroid cells. Expression of this CD164+CD33+ subset is also found on developing myelomonocytic cells in bone marrow, being downregulated on mature neutrophils but maintained on monocytes in the peripheral blood. We have extended these studies further by identifying artificial chromosome (PAC) clones containing the CD164 gene and have used these to localize the CD164 gene specifically to human chromosome 6q21.

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part of an adhesion cascade. In such cases, the mucin receptor/selectin ligand interactions would mediate the initial weak tethering of leukocytes to endothelium that precedes stronger integrin-mediated adhesion and subsequent transendothelial migration required for leukocyte trafficking. Although mucin receptors may be widely expressed, their function may differ on different cell types or on the same cell type under different states of activation. This functional diversity is dependent on the core peptide of the mucin and on the cell-specific expression of sialomucins have a variety of functions, with the specificity in the inactivation of cell adhesion and the negative regulation of their growth and/or presentation of the O-linked oligosaccharide sidechains, membrane anchorage, signal transduction abilities, and/or the trafficking of the mucin to the correct cellular domain. These alterations may then affect function.

Although a great deal of research has been directed toward the expression and function of the mucins on mature leukocytes and endothelial cells, there is still a paucity of information about their expression and function on human CD34+/hematopoietic “stem” and progenitor cells and on the associated stromal and endothelial cells that constitute the immediate stem-cell microenvironment. Previous studies have identified three of the mucin-like receptors, CD34, PSGL-1, and CD43, on primitive human bone marrow hematopoietic progenitor cells and/or associated stromal/endothelial cells. More recently, we have identified and cloned a fourth sialomucin-like receptor, CD164, on human hematopoietic progenitor cells and bone marrow stromal reticular cells. On such cells, the four sialomucins have a variety of functions, with the specificity in receptor/ligand interactions depending on the structural characteristics of the mucin-like receptor. These functions include mediating, or regulating hematopoietic progenitor cell adhesion and the negative regulation of their growth and/or differentiation.

In this report, we have characterized four monoclonal antibodies (MoAbs) against the CD164 molecule. These MoAbs each recognize distinct epitopes on the CD164 molecule. We have examined the expression of these epitopes on CD34+ cells from cord blood and bone marrow. Because we have recently shown that antibody ligation of the 103B2/9E10 epitope can inhibit the proliferation of single human CD34+CD38dim− hematopoietic progenitor cells in the presence of the cytokines, interleukin-3 (IL-3), IL-6, granulocyte colony-stimulating factor (G-CSF), and Steel factor in serum free medium and the adhesion of CD34+ cells to bone marrow stromal reticular cells, we also analyzed the distribution of this epitope on subsets of human bone marrow and cord blood cells and on their CD34+ cell precursors in some detail. Our results show that this epitope is present on phenotypically very primitive CD34+ progenitor cells, as defined by their coexpression of such surface antigens as AC133, Thy-1, FLT3 receptor, and c-kit receptor, but it exhibits differential expression on maturing B cells. Furthermore, using the CD164 cDNA as a probe, we have isolated PAC clones containing the CD164 gene and have used these to show, by fluorescence in situ hybridization (FISH) analysis, that the CD164 gene is localized to human chromosome 6q21.

MATERIALS AND METHODS

Cells and Cell Lines

Cell lines were cultured in RPMI 1640 or Iscove’s modified Dulbecco’s medium (IMDM) containing 10% to 15% (vol/vol) fetal calf serum (FCS; GIBCO-BRL, Paisley, Scotland). Heparinized human umbilical cord blood, normal peripheral blood, and normal bone marrow were drawn with informed consent of donors and with ethical permission from the Department of Transplantation Sciences, Southmead Hospital, Bristol, from local institutes or from the Medical Clinic, University of Tübingen, Tübingen, Germany.

Cell Isolation and Erythroid Cultures

Fresh human bone marrow, cord blood, and peripheral blood samples were fractionated on Ficoll-Hypaque (1.077 g/mL; Sigma Chemical Co, St Louis, MO). The light density cells were collected and any contaminating erythrocytes lysed in 0.147 mol/L NH4Cl for 30 to 60 minutes at room temperature. CD34+ cells were isolated using the Miltenyi Biotech (Bergisch Gladbach, Germany) Mini-MACS CD34 stem cell isolation kit as specified by the manufacturer. Cells were resuspended and cultured at 3,000 to 5,000 cells per mL in 24-well Falcon 3047 tissue culture plates (Becton Dickinson, Sunnyvale, CA) in defined StemBio-A. Erythroid-CNRs serum-free media (StemBio-CNRs, Villejuif, France) containing optimal concentrations of the cytokines, IL-3, IL-6, Steel factor, and erythropoietin in a humidified incubator at 37°C in the presence of 4.5% (vol/vol) CO2 and 5% (vol/vol) O2 gas mixtures. Cells were obtained on days 5, 6, 9, and 13; the nucleated cell number determined; and cytopsins photographed after centrifugation onto slides as described below. In some instances, the cells were stained with antibodies and analyzed on the FACSCalibur (Becton Dickinson).

Peripheral blood granulocytes (neutrophils) were isolated from 1 mL of Ficoll-hypaque–pelleted cells (density >1.077 g/mL) after a 20-second erythrocyte lysis with 20 mL of distilled water. After mixing, 20 mL of 0.308 mol/L ice-cold NaCl was added and the cells washed twice before staining.

Generation and Characterization of CD164 Antibodies

The CD164-specific MoAbs, 103B2/9E10 and 105A5, were generated after the immunization of mice with the erythromegakaryocytic cell line, MOLM-1; antibody 67D2 by immunization with the breast carcinoma cell line T47-D (obtained from the American Tissue Culture Collection); and antibody N6B6 by immunization with the pre-B-cell line Nalm-1 (obtained from the German Collection of Microorganisms and Cell Cultures), according to previously described methods. The 103B2/9E10 and 105A5 MoAbs were used for expression cloning. Comparative analysis revealed that both MoAbs recognized isolated FDCP-1 transfectant cell lines expressing human CD164. A detailed description of the isolation, sequencing, and generation of CD164 transfectant cell lines is described separately. In addition, the 67D2 and N6B6 MoAbs were also found to specifically recognize murine FDCP-1 cells expressing human CD164 but not the parental FDCP-1 cells (see Fig 1).

Antibodies and Antibody Conjugates

The isotypes of the MoAbs were determined by enzyme-linked immunosorbent assay (ELISA) (Boehringer-Mannheim, Mannheim, Germany). The murine 103B2/9E10 (IgG1, isotype), 105A5 (IgM isotype), 67D2 (IgG1, isotype,) and N6B6 (IgG2a, isotype) MoAbs to human CD164 were used as culture supernatants or purified Ig preparations. The mouse anti-human CD34 MoAb (clone 43A1; IgG1) was generated by immunization with KG1A cells and then assigned to the CD34 cluster as described by Greaves et al. For immunohistochemistry, mouse MoAb to human CD3 (clone 3D4; IgG1), glycophorin A (clone FC159; IgG1), glycophorin C (clone Rmt10; IgG1), and band III
(clone Q1/156; IgG1) were purchased from Dakopatts (Copenhagen, Denmark) as culture supernatants or purified Ig fractions. For dual- and multicolor fluorescence-activated cell sorter (FACS) analysis, the following mouse antibody conjugates, coupled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or PerCP were used: (1) CD3-PE or -FITC (clone SK7; IgG1); CD4-FITC (clone SK3); CD8-FITC (clone SK1; IgG1); CD14-FITC (clone MOP9; IgG3); CD20-PE or -FITC (clone L27; IgG1); CD19-PE or -FITC (clone SJ25C1; IgG1); HLA-DR-PE (clone L243; IgG2α); CD34-FITC, -PE, or -PerCP (clone 8G12; IgG1); CD38-PE (HB-7; IgG1); and CD33-PE (clone p67.6; IgG1; all from Becton-Dickinson, San Jose, CA); (2) CD117-PE (clone 95C3; IgG2a); CD65-FITC (clone 88H7; IgG1); CD66b-FITC (clone 80H3; IgG1); and anti-glycophorin A-PE (clone 11E4B7; IgG1; all from Immunotech, Marseille, France). (3) CD90-PE (clone 5E10; IgG1; Pharmingen, Hamburg, Germany); (4) CD71 (clone T5614; IgG1; Biorend, Cologne, Germany); and (5) AC133-PE (clone AC133; IgG1; Amecell Corporation, Sunnysvale, CA). The CD135-PE (FTL3; clone SF1.340; mouse IgG1) was a kind gift from Drs O. Rosnet and A. van Aghoven, Marseille, France. 

As comparative negative controls, irrelevant antibodies (Dakopatts, Immunotech, or Becton Dickinson) of the same isotypes and with equivalent fluorescent tags or phosphate-buffered saline (PBS) were used in place of primary antibodies. Texas Red (TR), FITC-, or PE-conjugated isotype-specific secondary antibodies were purchased from Southern Biotechnology Associates Inc, Birmingham, AL, and FITC (Fab)2, goat or rabbit anti-mouse Ig from Dianova, Hamburg, Germany or Dakopatts. All antibodies were used at 5 to 10 µg/mL per 107 cells/mL or at the concentrations recommended by the manufacturer.

**Immunofluorescence Staining for Flow Cytometric Analysis and Cell Sorting**

Single-color staining. Cells were blocked with 10% (vol/vol) human AB serum (Behring, Marburg, Germany) or human gamma globulin (30% [vol/vol] FC blocking reagent; Mitenyi Biotech) for 10 to 20 minutes at 4°C and then labeled with saturating concentrations of culture supernatants of the CD164 MoAbs, 103B2/9E10, 105A5, 67D2, or N6B6 MoAbs and counterstained with PE-conjugated goat anti-mouse isotype-specific Ig or with FITC-anti-mouse Ig. Peripheral blood mononuclear cells (see Fig 9) were labeled with 103B2/9E10 or an irrelevant IgG1- control antibody, together with CD4-FITC, CD6-FITC, CD3-FITC, CD20-FITC, or irrelevant FITC-isotype-matched controls before counterstaining with PE-conjugated anti-mouse IgG1.

**Flow cytometric analysis and cell sorting.** Cells were analyzed on a FACS Calibur using Cellquest software or analyzed and sorted on a FACS Vantage flow cytometer (all from Becton Dickinson). The fluorescence of FITC, PE, and PerCP was excited with an argon ion laser at 488 nm and detected at emission wavelengths of 530 nm, 570 nm, and 670 nm, respectively. Cell sorting of CD34+CD164+ (103B2/9E10 epitope) and CD34+CD164+ (103B2/9E10 epitope) bone marrow cells was performed at 25 kV using FITC-CD34 and the 103B2/9E10 MoAb followed by PE-conjugated anti-mouse IgG1. Sorted fractions were cyt centrifuged and stained with May-Grünwald-Giemsa solution for morphological analysis.

**Dual-Color Immunofluorescence of Cytospins**

All incubations were performed at room temperature for 30 minutes. After Fc receptor blockade as above, cytospins were incubated with 103B2/9E10, 105A5, or N6B6 MoAbs followed by FITC-conjugated isotype-specific secondary antibodies (1:25 dilutions in PBS). After washing in PBS, cells were incubated with CD3 (as a negative irrelevant IgG1 control), anti-glycophorin A, anti-glycophorin C, or anti-β3 and developed with TR-conjugated goat anti-mouse IgG1 (1:50 dilution in PBS). The slides were mounted in fluorescent mounting medium (Dakopatts) containing 2% (wt/vol) 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) and viewed under an Olympus BX-60 fluorescence microscope (Olympus, London, UK).

**Cross-Blocking Analysis of CD164-Specific MoAbs**

For epitope mapping studies, MOLT-1 cells were incubated with either 103B2/9E10, 105A5, 67D2, or N6B6 MoAbs (all different isotypes), or with isotype-matched negative control antibodies all at concentrations of 5 µg/mL at 107 cells/mL for 30 minutes on ice. After washing, cells were incubated either with the same MoAbs to indicate positive or negative controls, respectively, or with test CD164 MoAbs that differed from the blocking MoAbs. In the final step, cells were stained with PE-conjugated anti-Ig that specifically identified the CD164 test MoAb and analyzed on a FACS Calibur flow cytometer. The percent blocking was calculated as follows: 100 × [(Median Fluorescence of Cells Stained With Test MoAb After Incubation with Blocking MoAb) – (Median Fluorescence of Cells Stained With Isotype-Matched Negative Control MoAb)] ÷ [(Median Fluorescence of Cells Stained With Test MoAb After Incubation With Negative Control MoAb) – (Median Fluorescence of Cells Stained With Isotype-Matched Negative Control MoAb)] × 100.

**Sensitivity of CD164 Epitopes to Vibrio cholerae Neuraminidase Treatment**

Calu-1, KG1A, MOLT-1 and BV-173 cells were incubated with 0.2 U/mL of neuraminidase from *V. cholerae* (Calbiochem, Heidelberg, Germany) for 60 minutes at 37°C in 250 mL PBS. After washing with ice-cold staining buffer, cells were blocked and labeled with each of the CD164 MoAbs or the appropriate negative isotype-matched control MoAb and counterstained with PE-conjugated isotype-matched anti-Ig. Cells were analyzed on a FACS Calibur, and percent binding was calculated as follows: [(Median Fluorescence of Neuraminidase-Treated Cells Stained With CD164 MoAb) – (Median Fluorescence of Neuraminidase-Treated Cells Stained With The Isotype-Matched Negative Control MoAb)] ÷ [(Median Fluorescence of Untreated Cells Stained With CD164 MoAb) – (Median Fluorescence of Untreated Cells Stained With The Isotype-Matched Negative Control MoAb)] × 100.

**CD164 cDNA and Probes**

Two human CD164 cDNA clones (clone 105A5 and 103B2), isolated from a retroviral cDNA library of human bone marrow stromal cells by expression cloning with the 105A5 and 103B2/9E10 MoAbs, and subcloned into the pGEM-T vector (Promega, Southampton, UK) were transformed into XL2 Blue-MRF' bacteria (Stratagene, Cambridge, UK). Large-scale DNA preparations of the two CD164 cDNA clones were purchased from Stratagene (Copenhagen, Denmark) as culture supernatants or purified Ig fractions.
were purified on CsCl gradients,\textsuperscript{41} completely sequenced\textsuperscript{3,5} using oligonucleotide primers, and used to probe genomic libraries. The nucleotide and the predicted peptide sequences have been described previously.\textsuperscript{7} Initial screening of a human PAC library was performed with two CD164 cDNA probes, A and B, derived from the 105A5 cDNA clone in the pGEMT vector (Promega). The Sac I/Spe 1 probe A fragment contained 1 to 1907 bp of cDNA sequence, where bp1 indicates the translational start site. This encompasses the whole translated sequence plus part of the 3'UTR. The Spe I/Apa 1 probe B fragment comprised 1908 to 2867 bp of 3'UTR. Subsequent screening of the human PAC subclones and of Southern blots was performed with a 1.173 kb EcoRV/Hind III CD164 probe from the 105A5 cDNA (Probe C), which contained CD164 cDNA sequence, including a region spanning 1309 to 2487 bp of untranslated CD164 sequence in the 3'UTR. The restriction enzymes were purchased from Boehringer-Mannheim and endonuclease digestions performed in the buffers supplied with the enzymes and according to the manufacturer’s instructions. Restriction fragments were isolated after electrophoresis on 1.5% to 2% (wt/vol) NuSieve agarose (FMC Bioproducts, Rockland, ME) in TAE buffer (40 mmol/L Tris-acetate buffer, pH 8.5, containing 2 mmol/L EDTA) and purification on Wizard PCR (polymerase chain reaction) prep columns (Promega) as detailed by the manufacturer. DNA concentrations were determined by agarose gel electrophoresis against known phase DNA quantitation standards (GIBCO-BRL). For labeling, 20 to 50 ng of each cDNA probe was labeled with 50 µCi α-\textsuperscript{32}P-dCTP (Amersham Int, High Wycombe, Bucks, UK) using the T7 Quickprime kit (Pharmacia, Uppsala, Sweden) according to the manufacturer’s protocol.

**Fig 1.** Binding of CD164 MoAbs to stable FDCP-1 cells expressing CD164. FDCP-1 cells stably transfected with CD164 cDNA were labeled with the N6B6, 67D2, 105A5, or 103B2/9E10 MoAbs or with irrelevant first MoAbs of the same isotype. The reaction was developed with FITC-conjugated anti-mouse Ig antibody as detailed in Materials and Methods, and the median fluorescence values were determined after FACSCalibur analysis. The results of one of three experiments are shown. The IgG negative control histogram contains 100% IgG fluorescence. Median fluorescence intensities for the negative isotype matched controls were: no first MoAb, 23.9; 67D2, 13.5; N6B6, 21.4; and 103B2/9E10, 11.1. Percent blocking was calculated from median fluorescence intensities as indicated in Materials and Methods.

**Table 1. Epitope Mapping of CD164-Specific MoAbs**

<table>
<thead>
<tr>
<th>Test CD164 MoAb</th>
<th>Binding CD164 MoAb</th>
<th>Blocking CD164 MoAb</th>
<th>Exon 1</th>
<th>Exons 1 and 2</th>
<th>Exons 1, 2, and 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>103B2/9E10</td>
<td>—</td>
<td>0</td>
<td>3.5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>105A5</td>
<td>—</td>
<td>0</td>
<td>3.5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N6B6</td>
<td>60.4</td>
<td>—</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>67D2</td>
<td>—</td>
<td>21.4</td>
<td>90.1</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>13.5</td>
<td>3.5</td>
<td>98.3</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

MOLM-1 cells were stained with one of the unconjugated CD164-blocking MoAbs or with the appropriate isotype-matched negative control MoAb before labeling with the second test CD164 MoAb, which differed from the blocking MoAb. After staining these cells with PE-conjugated anti-lg that specifically reacted with the CD164 test MoAb, they were analyzed on the FACSCalibur and their median fluorescence intensities determined. Percent blocking was calculated from median fluorescence intensities as indicated in Materials and Methods.

\*Soluble domain deletion constructs, derived from exons 1; 1 and 2; and 1, 2, and 3 were prepared after determination of the intron-exon structure of the CD164 gene,\textsuperscript{7} subcloned into the pEFBoa-ig mu vector,\textsuperscript{4} and expressed in 293T cells as soluble recombinant proteins as detailed elsewhere.\textsuperscript{4} The predicted peptide sequences for these soluble constructs are: Exon 1: D[\textsuperscript{24}K][\textsuperscript{29}N][\textsuperscript{138}T]G[\textsuperscript{142}P][\textsuperscript{147}V]P[\textsuperscript{152}P][\textsuperscript{157}P]; Exon 2: ЕТСГРИСЦСФЦ[С]Н5ВТТЦФ1ВИЕВ; Exon 3: DESYCHSTVSDQCV[Г]NTTFC[С]S with exon 1 predicted to contain nine O-linked glycosylation sites on the serine and threonine residues underlined. Potential N-linked glycosylation sites are indicated in bold lettering. The data represent a summary of the results of CD164 MoAb binding to these soluble recombinant proteins as determined by ELISA assays.\textsuperscript{6}

**Isolation and Subcloning of PAC Clones**

The human PAC library derived from normal human male genomic DNA in the PCYPAC2N vector, containing 120,000 clones and grided on to 7 Hybond N filters, was kindly provided by the HGMP Resource Centre (Cambridge, UK) via Dr P. de Jong’s group. Filters were hybridized with the α-\textsuperscript{32}P-labeled human CD164 probes A and B as described below. Five identified positive clones were obtained from the HGMP Resource Centre and grown overnight in 2× TY broth containing 25 µg/mL kanamycin. These were digested with EcoRI, electrophoresed on a 0.7% (wt/vol) agarose gel, Southern blotted, and probed with α-\textsuperscript{32}P--labeled CD164 probe C or analyzed by PCR using forward MGC-GP-F3 and reverse MGC-GP-B3 oligonucleotide primers (Oswell, Southampton, UK) and the PCR products sequenced as described below. The MGC-GP-F3 (455-479) and MGC-GP-B3 (1601-1757) primers were 5’-CCTCACAACCTGTGCGAAAGTCTAC3’ and 5’-ACTCAAGACAGTCTGGTGG AAA TCC-3’. The restriction enzymes were purchased from Boehringer-Mannheim and endonuclease digestions performed in the buffers supplied with the enzymes and according to the manufacturer’s protocol. The PCScript (SK\textsuperscript{+}) vector was digested with Pst I or BamHI for 1 hour at 37°C in the appropriate buffers before the addition of 1 µL of shrimp alkaline phosphatase (Boehringer Mannheim) for 1 hour at 37°C. After heat inactivation at 68°C for 15 minutes, the enzyme-digested vector was...
CD164 EXPRESSION AND CHROMOSOME LOCATION

Table 2. Sensitivity of CD164 Epitopes to Sialidase

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>% CD164 MoAb Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD164 MoAb</td>
<td>103B2/9E10 105A5 N6B6 67D2</td>
</tr>
<tr>
<td>Calu-1</td>
<td>93.8 ± 5.6 18.8 ± 1.1 88.9 ± 10.2 118.5 ± 1.5</td>
</tr>
<tr>
<td>KG1A</td>
<td>74.8 ± 8.7 0 ± 0 85.2 ± 6.4 94.6 ± 4.4</td>
</tr>
</tbody>
</table>

Calu-1 and KG1A cells were incubated with 0.2 U/mL of Vibrio cholerae sialidase for 60 minutes at 37°C. Cells were then washed and stained with each CD164 MoAb indicated or with an isotype-matched negative control MoAb and counterstained with PE- or FITC-conjugated isotype-matched anti-Ig. The median fluorescence for each cell group was determined by analysis on a FACSCalibur, and the percent binding determined as indicated in Materials and Methods. Values are means ± SD of three determinations.

DNA extracts from human x hamster or human x mouse somatic cell hybrids were kindly provided by the HGMP Resource Centre, Cambridge, UK. These (100 ng) were analyzed by PCR using the MGC-GP-F3 and MGC-GP-B3 primer pairs described above or the MGC-GP-F4 (1566-1591) and MGC-GP-B4 (2069-2045) primer pairs, 5'-GCTCCTTGAAGGAA TTTCCACCAGAC-3' and 5'-CAATGCG- GAAACTCAGCCACTA TTG-3', respectively (all from Oswell, Southampton, UK) to the CD164 cDNA sequence. PCR analysis was also performed on two human male and female genomic DNA preparations, human genomic DNA provided with the hybrid panel, the BglII subclone of CD164 PAC1, and the CD164 cDNA clones, 103B2/9E10 and 105A5. The PCR on the somatic cell hybrid panel was performed with 100 ng of each chromosome 1-22; chromosome X; chromosome Y DNA; plus mouse, human, and hamster DNA controls using the Expand Long Template System (GIBCO-BRL) and the following program: a hotstart of 95°C for 2 minutes, followed by 30 cycles of 95°C for 1 minute for denaturation, 65°C for 30 seconds for annealing, and 69°C for 4 minutes.
for extension; and a final extension cycle of 69°C for 10 minutes. Essentially the same protocol was used for PCR analysis of the PAC clones and subclones and the CD164 cDNAs. PCR analysis was, however, performed on the human genomic DNA samples using the Advantage KlenTaq System (Clontech, Palo Alto, CA) and the following PCR program used: a hotstart of 95°C for 2 minutes, followed by 5 cycles of 95°C for 30 seconds and 72°C for 3 minutes, 5 cycles of 95°C for 30 seconds and 68°C for 3 minutes, and a final extension cycle of 68°C for 7 minutes. The PCR products (10 µL) were analyzed by 2% (wt/vol) agarose gel electrophoresis in Tris borate EDTA (TBE) buffer using a 1-kb DNA ladder (GIBCO-BRL) as the molecular weight marker and blotted onto Nytran-N Nylon membranes (Schleicher and Schuell, Dassel, Germany). These membranes were prehybridized in 30 mL of 50% (wt/vol) formamide, 4× SSC, 0.001 mol/L sodium phosphate buffer (pH 7.2), 8% (wt/vol) dextran sulfate, 100 µg/mL of sonicated Herring sperm DNA (Sigma), and 25 µg/mL yeast tRNA (Sigma), 10× Denhardt’s solution46 and 50 µg human placental DNA (Sigma) at 42°C for 1 to 18 hours before addition of the α-32P–labeled CD164 probes A, B, or C for 18 to 48 hours at 42°C. Filters were washed twice at 42°C in 2× SSC with 0.1% (wt/vol) sodium dodecyl sulfate (SDS) for 30 minutes each, 3 times at 65°C in 0.2× SSC containing 0.1% (wt/vol) SDS for 45 minutes each, and then exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY) with intensifying screens at −70°C as described.46

**Automatic Sequencing**

Miniprep or CsCl gradient prepared DNA (200 µL) was denatured at 37°C for 30 minutes with 20 µL 2 mol/L NaOH and 2 mmol/L EDTA. Automatic Sequencing

Miniprep or CsCl gradient prepared DNA (200 µL) was denatured at 37°C for 30 minutes with 20 µL 2 mol/L NaOH and 2 mmol/L EDTA.

**Fig 3.** Differential expression of CD164 epitopes on CD34+ bone marrow and cord blood cells from normal donors. Mononuclear cells were stained with CD164-specific MoAbs 103B2/9E10, 105A5, 67D2, and N6B6 together with CD34 as indicated in Materials and Methods and gated on forward and side scatter (top dot plots) before analysis on a FACSCalibur flow cytometer. In four independent bone marrow and three independent cord blood analyses, 4.6 ± 0.8% and 1.2 ± 0.1% of the scatter gated cells were CD34+, respectively. In the representative experiment shown, median fluorescence values for the CD34+CD164+ subsets of the human bone marrow (BM) gated cells were: N6B6 = 138.24; 103B2/9E10 = 212.88; 105A5 = 45.32; 67D2 = 198.10 and for the CD34+CD164+ gated subsets were: N6B6 = 294.97; 103B2/9E10 = 184.34; 105A5 = 220.67; 67D2 = 697.83. Median fluorescence values for the CD34+CD164+ subsets of human cord blood (CB) cells were: N6B6 = 148.55; 103B2/9E10 = 294.27; 105A5 = 37.86; 67D2 = 283.87 and for the CD34+CD164+ gated subsets were: N6B6 = 119.71; 103B2/9E10 = 69.78; 105A5 = 77.74; 67D2 = 273.84. Cells were also labeled with CD34-FITC or the CD34 MoAb, 43A1, plus an anti–IgG3-FITC secondary antibody, together with isotype-matched irrelevant control MoAbs for each CD34+ MoAb, 43A1, plus an anti–IgG3-FITC secondary antibody. Under these conditions, 0.08% to 0.12% of cells occurred in the CD34+ Ig Isotype+ gates, and 0.08% to 0.38% of cells occurred in the CD34+ Ig Isotype+ gates.
and then ethanol precipitated. In some cases, the PCR products were directly sequenced after PEG precipitation. The PCR products (4 µL) or the DNA (0.5 µg) in 5 µL sterile double-distilled water were added to 4 µL of ABI Prism Ready Dye deoxy Terminator mix (Applied Biosystems, Perkin Elmer, Foster City, CA) with 0.025 µg M13 (Stratagene) or 3 pmol of CD164 forward or reverse primers (Oswell) in 2 to 3 µL sterile double-distilled water, and the sequencing reaction was performed according to the manufacturer’s protocol before analysis on an ABI 373 Automatic Sequencer (Applied Biosystems). The sequences were analyzed using MacVector, Seqed, Assemblign, Analysis, and Sequencher software packages (Oxford Molecular, Oxford, UK), aligned to each other and to the CD164 cDNAs and a contig generated.

**FISH**

Metaphase spreads were prepared from phytohemagglutinin-stimulated normal human lymphocytes by use of standard techniques. Before hybridization, the slides were denatured in 70% (vol/vol) formamide and 2× SSC at 73°C for 3 minutes, then washed in 2× SSC and dehydrated through an ethanol series of cold 70% (wt/vol), 95% (wt/vol), and absolute ethanol. The BglII and PstI subcloned PAC DNA in the pCRScript vector was biotinylated using the Bionick kit (GIBCO-BRL). Two hundred nanograms labeled probe was mixed with 5 µg Cot-1 DNA (GIBCO-BRL), precipitated, resuspended in 11 µL hybridization mix, denatured at 85°C for 5 minutes, and allowed to preanneal at 37°C for 30 minutes. The probe was then applied to a denatured slide and hybridized overnight. Slides were washed in 50% (vol/vol) formamide, 2× SSC pH 7 at 42°C, followed by 1× SSC at 60°C. Blocking solution (3% [wt/vol] BSA, 4× SSC and 0.1% [vol/vol] Tween 20) was applied and slides incubated at 37°C for 30 minutes. After incubation, avidin-FITC (diluted in 1% [wt/vol] BSA, 4× SSC, 0.1% [vol/vol] Tween 20) was applied and slides incubated at 37°C for 40 minutes. Slides were washed in 4× SSC, 0.1% (vol/vol) Tween 20 at 42°C and counterstained with 200 ng/mL DAPI, followed by 2 minutes in 2× SSC. Slides were mounted in Citifluor and images captured using a Photometrics KAF 1400-500 CCD camera (Photometrics, Tuscan, AZ) attached to a Zeiss Axioskop (Zeiss, New York, NY) epifluorescence microscope. Separate images of probe signals and DAPI banding patterns were pseudocoloured and merged using Smart-Capture software (Vysis Inc, Chicago, IL).

**RESULTS**

The MoAbs 103B2/9E10, 105A5, 67D2, and N6B6 Specifically Detect CD164 and Recognize Distinct CD164 Epitopes

The predicted amino acid sequence of cDNA clones selected by expression cloning of a human bone marrow stromal cell cDNA library with the CD164 MoAbs, 103B2/9E10 and 105A5, were identical. The complete sequence is described separately. Parental FDCP-1 cells or FDCP-1 cells expressing these human CD164 cDNAs were stained with 67D2 and N6B6 MoAbs, as well as with 103B2/9E10 and 105A5, and analyzed by flow cytometry. All four MoAbs selectively recognized CD164 cDNA transfected, but not parental, FDCP-1 cells (Fig...
1). Our more recent data indicate that the identified CD164 cDNA is encoded by 5 separate exons, with the 103B2/9E10 and 105A5 MoAbs reacting with soluble recombinant protein derived from exon 1. In contrast, the N6B6 and 67D2 MoAbs do not react with soluble recombinant proteins derived from exon 1 or exons 1 and 2, but bind to a recombinant protein comprising the extracellular domains encoded by exons 1, 2 and 3, as summarized in Table 1. Detailed epitope analyses using these and other soluble CD164 recombinant constructs are described elsewhere. Reactivities of the four MoAbs with cell surface–expressed epitopes of CD164 were analyzed in more detail on a set of hematopoietic and nonhematopoietic cell lines (data not shown). Because all four MoAbs reacted strongly with the MOLM-1, Calu-1, KG1A, and BV-173 cell lines, these were used in further experiments. Cross-blocking experiments were performed to determine whether the 103B2/9E10 and 105A5 or the N6B6 and 67D2 MoAbs recognized identical or different epitopes on cell lines. As indicated in Table 1, prelabeling of MOLM-1 with the 103B2/9E10 and 105A5 MoAbs did not substantially block 67D2 or N6B6 binding, nor did 105A5 MoAb prelabeling prevent the 103B2/9E10 MoAb from binding. However, 67D2 almost completely blocked N6B6 staining, and vice versa. Partial blocking of 105A5 was observed after 103B2/9E10 prelabeling, but not vice versa. This partial inhibition might be caused by conformational changes of the CD164 molecule potentially induced by the 103B2/9E10 MoAb. These results support those obtained for exon mapping and show a close association between the 103B2/9E10 and 105A5 epitopes and between the 67D2 and N6B6 epitopes.

**Sensitivity of CD164 Binding to Sialidase Treatment**

To further characterize CD164 epitopes, Calu-1 and KG1A cells were treated with *V. cholerae* sialidase, an enzyme that selectively hydrolyzes N- or O-acyl-neuraminic acids, which are α2,3-, α2,6- or α2,8-linked to galactose, hex, NAc, or N- or O-acylated neuraminyl residues in oligosaccharides, before staining with the CD164 MoAbs. Table 2 shows that the epitopes detected by the 103B2/9E10, N6B6, and 67D2 MoAbs are relatively resistant to desialylation compared with the untreated positive controls, whereas binding of 105A5 is almost completely abrogated. Preliminary results also indicate that the 105A5 MoAb (in contrast to the other three CD164 MoAbs) does not bind to sialidase treated MOLM-1 and BV-173 cell lines, with labeling being reduced to 7.8% and 0%, respectively, of the untreated cells. These sialidase studies are in line with the exon localization and cross-blocking studies in showing that, although the epitopes recognized by the 103B2/9E10 and 105A5 MoAbs are closely associated, they are distinct from one another and are different from those recognized by N6B6 and 67D2.

**Differential Expression of CD164 Epitopes on Normal Adult Peripheral Blood Cells and on Normal Bone Marrow and Cord Blood CD34⁺ Cells**

Staining of Ficoll separated peripheral blood cells from normal donors with the CD164 MoAbs 103B2/9E10, 105A5, 67D2, and N6B6 and the separation of cell subsets based on forward and side scatter parameters revealed that peripheral blood lymphocytes were weakly cell-surface positive with 103B2/9E10, 67D2, and N6B6, whereas staining with the 105A5 MoAb resulted only in faint signals near background staining. Monocytes generally showed higher levels of CD164 MoAb binding than did lymphocytes. Median fluorescence intensity (MFI) values for CD164 staining of monocytes were...
28.9 ± 6.8 for N6B6, 41.5 ± 15.6 for 67D2, 22.3 ± 27.1 for 103B2/9E10, and 7.5 ± 4.8 for 105A5, after subtraction of the MFI values for isotype-matched negative controls and where the values are means ± SD of three independent experiments. This compares with MFI values for lymphocyte staining of 7.9 ± 3.4 for N6B6, 8.3 ± 2.7 for 67D2, 2.7 ± 2.0 for 103B2/9E10, and 3.6 ± 2.0 for 105A5. Mature non-nucleated erythrocytes showed negligible staining with all four CD164 MoAbs (data not shown). Granulocytes were very weakly stained by the CD164 MoAbs, except for 105A5, which was completely negative. The relevant fluorescence profiles and MFI values of a representative experiment are shown in Fig 2.

The expression of the four CD164 epitopes was significantly higher on bone marrow and cord blood CD34⁺ cells than on peripheral blood mononuclear cells. When CD34⁺ cells were analyzed, staining was more consistent with the N6B6 and 67D2 MoAbs. In four independent experiments, 81.8 ± 9.9% and 81.5 ± 9.9% of CD34⁺ bone marrow cells expressed these respective epitopes. This is in contrast to more variable staining with the 103B2/9E10 and 105A5 MoAbs with 70.2 ± 26.3% and 63.3 ± 26.1% of CD34⁺ cells labeling, respectively. CD34⁺ bone marrow cells expressing the 103B2/9E10 epitope of CD164 exhibited the highest MFI values. Examples of dot plots and MFI values for the CD34⁺CD164⁺ cell subsets defined with the different CD164 MoAbs are shown in Fig 3.

Three-color immunofluorescence analysis indicated that the CD34⁺CD164⁺(103B2/9E10)⁺ bone marrow cells coexpressed the N6B6 and 67D2 epitopes (data not shown). Some differential cell-surface staining with the CD164 MoAbs was also observed on cord blood. When cord blood mononuclear cells were dual-labeled with CD34 and the different CD164 MoAbs (Fig 3), approximately 90% of the CD34⁺ cells expressed the 103B2/9E10 (92.6 ± 2.8%), 67D2 (88.7 ± 1.0%), and N6B6 (89.1 ± 8.9%) epitopes, whereas only 55.0 ± 11.6% of these
cells were 105A5 epitope⁺, where values are means ± SD of three independent experiments. Again, the MFI values obtained for the CD34⁺CD164⁺ cord blood subset were higher when the 103B2/9E10 epitope was detected than for the other CD164 reactive epitopes (Fig 3).

The CD34⁺CD164⁺ Subset Contains Phenotypically Primitive Cells

We have recently shown that the 103B2/9E10 epitope is expressed by clonogenic progenitors, pre-colony-forming unit-granulocyte-macrophage (pre-CFU-GM), colony-forming unit-granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM), burst-forming unit–erythroid (BFU-E), and granulocyte-macrophage colony-forming cells (GM-CFC), and that exogenous addition of the 103B2/9E10 MoAb to single CD34⁺CD38⁻ cells cultured in serum-free media in the presence of IL-3, IL-6, G-CSF, and Steel factor can inhibit the proliferation of these cells by up to 65%. Within these cultures, 5 ± 3% of 103B2/9E10 treated cells compared with 14 ± 2% of cells treated with an irrelevant IgG, MoAb proliferated by day 3, whereas 23 ± 1% compared with 50 ± 6%, respectively, of these same cells proliferated by day 10 when 100 to 200 single cells were analyzed. The 103B2/9E10 MoAb also partially inhibited the adhesion of CD34⁺ cells to bone marrow stroma. To gain more insight into the phenotype of the most immature CD34⁺CD164⁺(103B2/9E10 epitope)⁺ subset, CD34⁺ bone marrow and cord blood cells were stained with 103B2/9E10 and with selected MoAbs that define primitive CD34⁺ cell subgroups. Representative dot plots of human bone marrow–purified CD34⁺ cells stained with these markers are shown in Fig 4. The most primitive CD34⁺ cells are thought to occur in the CD90(Thy1)⁺, CD117(c-kit)⁺, CD135(FTL-3)⁺, AC-133⁺, CD38⁻, CD33⁺, and CD71lo subsets. It was therefore of interest that 49.9 ± 10.9%, 88.7 ± 6.4%, 94.8 ± 3.8%, and 54.3 ± 17% of the CD34⁺CD164⁺(103B2/9E10 epitope)⁺ bone marrow cells expressed CD90, CD117, AC-133, and CD135, respectively, in four independent experiments. This represented greater than 90% of those CD34⁺ bone marrow cells that were CD90⁺ (96.9 ± 2.2%), CD117⁺ (98.3 ± 1.1%), AC133⁺ (96.9 ± 1.8%), and CD135⁺ (90.2 ± 10.2%). The CD34⁺CD164⁺(103B2/9E10)⁺ bone marrow cell subset also contained both HLA-DR⁺ and HLA-DR⁻ cells and the CD38lo subset (Fig 4). In these same studies, 10.3 ± 4.3% and 25.9 ± 16.7% of the CD34⁺ bone marrow cells were CD71lo and CD33⁻, respectively. Of these, an average of 65% of the CD71lo and 89% of the CD33⁻ expressed the CD164⁺ (103B2/9E10) epitope. Similar results (data not shown) were obtained with cord blood, with essentially all CD34⁺ cells that coexpressed CD90, CD117, AC133, and CD135 being CD164⁺(103B2/9E10) epitope⁺. These data indicate that CD164 is present on a very primitive hematopoietic CD34⁺ progenitor cell subset in addition to early clonogenic cells.

CD164 Expression Is Maintained on Nucleated Erythroid Cell Subsets, but Is Lost on Terminal Erythroid Differentiation

The localization of day-14 BFU-E within the CD34⁺CD164⁺ (103B2/9E10 epitope)⁺ fraction of human bone marrow is consistent with the finding that a proportion of CD34⁺CD164⁺(103B2/9E10 epitope)⁺ cells coexpress the CD33, CD117, HLA-DR, and CD71 markers (Fig 4). Because the CD164/103B2/9E10 epitope was present on the BFU-E but absent from the mature erythrocytes in peripheral blood, and as the whole CD34⁺ erythroid lineage from the proerythroblast stage to the erythroblast, normoblast, and mature non- nucleated erythrocyte stages can be defined by the regulated expression of three markers, CD71, CD117, and the erythroid specific marker glycoporphin A, we used these markers to determine at which stage in the erythroid lineage this CD164 epitope was lost. Within the CD34⁺ erythroid lineage, CD117 and CD71 are expressed on both the proerythroblasts and erythroblasts, with
glycophorin A appearing on some erythroblasts, being strongly expressed on the more differentiated normoblasts, and then maintained to the mature erythrocyte stage. Although CD71 is maintained on normoblasts and then lost from reticulocytes and mature erythrocytes, CD117 expression is lost at the normoblast stage. When human bone marrow cells were defined by the erythroid/lymphoid/blast cell light scatter parameters shown in Fig 5, 52.9 ± 9.2% of these cells were CD34+CD164(103B2/9E10 epitope)+, where values are means ±SD of four independent evaluations. When these cells were examined for their coexpression of erythroid associated markers, averages of 93%, 82%, and 8% of these cells were CD71+, glycophorin-A+, and CD117+, respectively, in two independent experiments (Fig 5). In addition, a mean of 88% of the glycophorin A+ nucleated erythroid cells within these bone marrow samples were 103B2/9E10+. Similar results with cord blood (Fig 5) confirmed that this prominent CD34+CD164+ cell population consists predominantly of nucleated erythroid cells, although most but not all glycophorin A+ nucleated cells express the 103B2/9E10 epitope. This was confirmed in a separate experiment by morphological analysis of sorted CD34+CD164+ cell populations, which revealed that a large proportion of these cells belonged to the erythroid lineage (9% lymphocytes, 13% monococytes, 1% promyelocytes, 49% polychromatic normoblasts, 10% basophilic normoblasts, 3% erythroblasts, and 13% proerythroblasts).

To analyze which stage of erythroid development the CD164(103B2/9E10) epitope was expressed, CD34-enriched human umbilical cord blood cells were expanded in liquid culture under conditions known to generate erythroid cells. Cells were collected at days 5, 6, 9, and 13 of culture and their total nucleated cell number determined. In two separate experiments, 100- to 200-fold and 400- to 600-fold expansions in cell numbers were observed in these cultures at days 9 and 13, respectively. Cells were then centrifuged onto slides for fixation and staining with the CD164 MoAbs and with the erythroid specific markers, glycophorin C, glycophorin A, and band III, with glycophorin C being expressed on BFU-E and proerythroblasts before the appearance of the other markers on erythroblasts and normoblasts. At day 5, the cultured cells were mostly glycophorin A, glycophorin C, or band III negative (>98.5%). However, these cells stained strongly in the Golgi region for all CD164 MoAbs (95% to 99%). By days 6 to 9 of culture, the majority of cells were glycophorin C positive (80 and 90%; Fig 6) and a subset of these had begun to express glycophorin A (42% and 61%) and band III (11% and 24%). Essentially all cells in these day-6 to -9 cultures maintained their expression of the different CD164 epitopes (>92%; Fig 6 and data not shown). It was of interest to note, however, that in these cultures only a very small proportion of these nucleated erythroid cells that were exclusively glycophorin A or band III positive either failed to express or expressed very low levels of the CD164 epitopes. The day-9 erythroid-cultured cells were also analyzed on the FACSCalibur to compare the levels of cell surface expression of the CD164 epitopes and those of the erythroid specific markers. As indicated in Fig 7, the cultured erythroid cells at day 9 could be divided into two subsets, both exhibiting low side light scatter characteristics, but with either low to medium (the R1 subset) or medium to high forward light scatter parameters (the R2 subset). Both subsets expressed glycophorin C in high amounts (histograms B and C). However, cells within the R1 or lower forward scatter gate expressed higher levels of
glycophorin A (histogram E) and band III (histogram H) than those in the R2 or higher forward scatter gate (histograms F and I, respectively). The R1 gated cells were essentially all glycophorin A positive, but could be separated into two subsets based on positive or negative expression of band III (histogram H). This suggested that cells within the lower forward scatter gate were more mature, but not fully developed, nucleated erythroid components. However, all subsets exhibited essentially the same surface fluorescence intensities when stained with the 103B2/9E10, as indicated in a typical experiment in Fig 7 (histograms J to L). Thus, the 103B2/9E10 epitope is evident before the expression of specific erythroid markers and is lost only at the late stages of erythroid differentiation.

**CD164 Is Differentially Expressed During B Lymphoid and Myelomonocytic Development**

It was of interest that the CD34+CD164(103B2/9E10)lo/2 cell population in bone marrow (Fig 8) consisted mainly of CD19+ B-cell precursors, suggesting that the most immature B-cell subset in bone marrow is CD164+. Developing B cells in bone marrow, however, were CD164(103B2/9E10)+ as evidenced in Fig 8, which further shows that 60% to 70% of CD34+ B cells expressing CD19 or CD20 surface molecules coexpress the CD164(103B2/9E10) epitope. In contrast, the majority of CD34+ T cells, in bone marrow, staining with the CD3 marker were found in the CD164(103B2/9E10)+− cell gate (Fig 8). To determine whether this CD164 epitope was maintained on peripheral blood lymphocyte subsets, three individual Ficoll separated peripheral blood samples were scatter gated on the lymphoid gate and analyzed by two-color FACS analysis for the B lymphoid marker, CD20, and for the T-cell markers, CD3, CD4, and CD8 (Fig 9). The majority of CD3+, CD4+, CD8+, and CD20+ cells fell within the CD164(103B2/9E10)+− gate. Analyses of the MFI indicated that for these three peripheral blood samples the CD20+ B cells showed lower levels of CD164(103B2/9E10) expression (MFI = 3.95 ± 0.29) than the CD3+ T cell subset (MFI = 6.69 ± 0.44). In addition, within the T-cell subset, the CD8+ cells (MFI = 4.11 ± 0.33) showed lower levels of CD164(103B2/9E10) epitope expression than did the CD4+ cells (MFI = 8.47 ± 0.12). Our other studies (data not shown) examining the correlated expression of the CD164(103B2/9E10) epitope and defined lymphoid surface markers on cord blood cells indicated that the majority of cord
CD164 Expression and Chromosome Location

Monocyte stages; the myelomonocytic marker, CD65; and CD14, which is expressed from the promonocyte to the more mature band forms and neutrophils in the neutrophilic lineage. Figure 10 confirms that essentially all CD14+ monocytes but is virtually absent from mature neutrophils in peripheral blood (Fig 2). We therefore used three cell-surface markers to analyze the expression of this CD164 epitope on CD34+ myelomonocytic cells in bone marrow. These were CD14, which is expressed from the promonocyte to the monocyte stages; the myelomonocytic marker, CD65; and CD66b, which exhibits high levels of expression on myelocytes and metamyelocytes and lower levels of expression on the more primitive promyelocytes and early myelocytes and on the more mature band forms and neutrophils in the neutrophilic lineage. Figure 10 confirms that essentially all CD14+ cells within the monocytic lineage and all except the more mature cells within the neutrophilic lineage are CD164(103B2/9E10)+.

Identification of CD164 Containing PAC Clones

A PAC library containing 120,000 recombinants was screened with full-length CD164 cDNA probes, and two positive clones (termed CD164 PAC1 and PAC5) were analyzed in detail. Restriction enzyme analyses of these clones, using EcoR1, BglII, Pst I, HindIII, and BamHI revealed that the pattern of bands on a 0.7% agarose gel stained with ethidium bromide was similar but not exactly identical (data not shown). When these gels were Southern blotted and hybridized with the 1.173 kb EcoRV/HindIII CD164 probe C, however, the pattern of bands observed for CD164 PAC1 was identical to that obtained with CD164 PAC5. Southern blotting of human placental genomic DNA digested with all five restriction enzymes and hybridized with CD164 cDNA probe C produced the same banding pattern (data not shown), indicating that the PAC clones had not undergone gross rearrangements within the region of the CD164 gene. A single hybridization band was observed with each enzyme, the smallest being an EcoR1 fragment of approximately 2.2 kb, whereas the largest fragment of greater than or equal to 18 kb was obtained after BamHI restriction digestion. When the blot was reprobed with the 5’ end of the cDNA, two additional BamHI fragments of 2 and 3 kb were identified. These three BamHI fragments cover the entire CD164 gene and span approximately 23 kb of DNA. Complete sequence analysis has been obtained from these BamHI fragments derived from the CD164 PACs after subcloning into pCRScript and these contain a sequence that is identical to the CD164 cDNA from 1 to 2867 bp, but they are interspersed with intronic sequences of varying sizes.52

PCR Analysis of Somatic Cell Hybrids Localizes CD164 to Chromosome 6

PCR analysis was performed using DNA from a set of somatic cell hybrids (Tables 3 and 4), from the BglII subclone of CD164 PAC1 and from three human genomic DNA samples. For the human genomic DNA, the CD164 PAC1 subclone and the hybrid containing human chromosome Xq13 translocated to 6p21, t(6,X)(p21,q13), single bands of approximately 1.1 kb and 500 bp were obtained with the MGC24-Gp F3/B3 and F4/B4 primers, respectively, on ethidium bromide-stained gels. These bands hybridized to the CD164 cDNA probe C. An example of the PCR products generated with the MGC24-Gp F4/B4 primers from the somatic cell hybrid panel is shown in Fig 11A and B. These PCR products were not generated with the hybrid DNA containing the X chromosome alone or in conjunction with chromosomes other than 6. This indicated that the CD164 gene was very likely to be located on chromosome 6. A sequence comparison of the products generated by PCR analysis of the hybrids revealed identical sequence for both the cDNA clones, the BglII subclones of the CD164 PACs, and genomic DNA from male or female peripheral blood leukocytes.
FISH Localizes CD164 to Chromosome 6q21

Sixty metaphase spreads were examined for signal using each of the BgIII subclones of CD164 PACS 1 and 5. Probe efficiency was approximately 60%, with signal being observed on both chromatids of chromosome 6 on band 6q21 only (Fig 11C).

DISCUSSION

The interaction of hematopoietic progenitor cells with stromal/endothelial cells in their immediate microenvironment is thought to be of major importance in the regulation of hematopoietic stem cell self-renewal, quiescence, commitment, and migration. These interactions involve cooperation between adhesion receptors, their cognate ligands, and cytokines. The adhesion receptors/ligands implicated in these processes belong to several families. These interactions involve cooperation between adhesion receptors, their cognate ligands, and cytokines. The adhesion receptors/ligands implicated in these processes belong to several families. These include the integrins, selectins, Ig superfamily, and sialomucins. Recently, mucin-like receptors or sialomucins, in particular, have assumed some importance in hematopoiesis, with four of these receptors, CD34, PSGL-1, CD43, and CD164, having been identified on primitive hematopoietic precursor cells and/or their associated stromal/endothelial elements.5,7,19-27 Our own studies and those of other researchers indicate that these receptors are involved in mediating or regulating hematopoietic precursor cell adhesion in vitro and/or the regulation of hematopoietic progenitor cell proliferation.5,7,20,31,32

Our own studies and those of other researchers indicate that these receptors are involved in mediating or regulating hematopoietic precursor cell adhesion in vitro and/or the regulation of hematopoietic progenitor cell proliferation.5,7,20,31,32

In this report, our studies have concentrated on the CD164 molecule. To this end, we have characterized the reactivity of the first four MoAbs, 103B2/9E10, 105A5, 67D2, and N6B6, to this sialomucin. Each of these antibodies shows distinct patterns of reactivity on CD34+ hematopoietic progenitor cells, labeling on average 55% to 93% of these cells. When compared with other CD164 epitopes, the 103B2/9E10 epitope is more highly expressed on CD34+ cells from both cord blood and bone marrow, whereas the 105A5 epitope exhibits the most variable level of expression. Our recent studies suggest that this may reflect glycosylation differences in the 105A5 and 103B2/9E10 subsets. We have cloned the CD164 cDNA5,7 and have shown that this gene encodes an 80 to 90 kD heavily O-glycosylated protein that is able to form homodimers with an apparent molecular weight of 160 to 180 kD. Our recent studies have also shown that the CD164 receptor mediates the adhesion of CD34+ hematopoietic progenitor cells to bone marrow stroma, since this adhesion can be partially blocked by the 103B2/9E10 MoAb.5,7 In addition, two-color phenotypic selection, based on expression of CD34 and the CD164(103B2/9E10) epitope, revealed that essentially all the committed myeloid and erythroid progenitors and their immature precursors were present in this CD164+ population and correspondingly absent from the CD34+CD164− subset. In contrast, mature peripheral blood cells showed only low or negligible levels of CD164 expression. In view of these results, we have characterized, in more detail, the expression of the 103B2/9E10 epitope on CD34+ and CD34+ subsets of cord blood and bone marrow cells. Multiparameter flow cytometric analyses revealed that the majority (>90%) of CD34+ human bone marrow and cord blood cells that expressed CD90(Thy-1),

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Hybrid: GM07299, 1 and X; MCP6BRA, Xpter-Xq13.6p21-6qter; CA4, 8 and fragment of 22; 762-8a, 10 and Y; 1aA9602 + ve, 12, 21 and X; 289, 13 plus fragments of 8, 11, and 12; GM10479, 14 plus part of 16 (probably 16p13.3-16q22.1); HORLI, 15, 11q, part of Xp and proximal Xq; GM10478, 20, 4 (part), 8 (part), 22q, and X; GM10612, 9p stronger than 9q, only 10% of cells with whole 9; PGME25NU, 22, part of Xp.

+ indicates presence of chromosome; − indicates absence of chromosome; / indicates chromosome translocation, extra chromosomes, or other modifications.
CD117(c-kit), AC-133, and CD135(FLT3) also coexpressed the 103B2/9E10 epitope. Because human AC-133 cells have the capacity for hematopoietic engraftment in fetal sheep, these results suggest that the 103B2/9E10 epitope is present on a very primitive subset of CD34<sup>+</sup> cells. Similarly, analyses of the CD34<sup>lo</sup>/CD164(103B2/9E10 epitope)<sup>+</sup> subsets have also indicated that one of the most prominent populations consists of nucleated erythroid cells, with the appearance of this CD164

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DNA samples were PCR amplified using CD164 primers as indicated in Materials and Methods and analyzed on 2% agarose gels. Positive bands of the correct size were obtained with the chromosome 6 DNA sample only. These products were Southern blotted and probed with CD164 cDNA Probe C or sequenced and were shown to have sequence identical to CD164 cDNA. −, no PCR product; +, PCR product of the appropriate size. Human, mouse, and hamster genomic DNA samples were also analyzed, but only the human DNA proved positive for CD164 under the conditions used.

CD117(c-kit), AC-133, and CD135(FLT3) also coexpressed the 103B2/9E10 epitope. Because human AC-133<sup>+</sup> cells have the capacity for hematopoietic engraftment in fetal sheep, these results suggest that the 103B2/9E10 epitope is present on a very primitive subset of CD34<sup>+</sup> cells. Similarly, analyses of the CD34<sup>lo</sup>/CD164(103B2/9E10 epitope)<sup>+</sup> subsets have also indicated that one of the most prominent populations consists of nucleated erythroid cells, with the appearance of this CD164.

Fig 11. CD164 is located on human chromosome 6q21. Somatic cell hybrids shown in Tables 3 and 4 were analyzed by PCR for the presence of the CD164 gene. (A) Ethidium bromide–stained gel of PCR products using the MGC24-Gp-F4/B4 primer pairs. This gel was Southern blotted and probed with CD164 cDNA probe C as indicated in (B). Partial metaphase spreads (C) showing localization of the Bgl II subclone of CD164 PAC1 to human chromosome band 6q21 (arrowed and shown on the idiogram).
epitope preceding the expression of the erythroid markers, glycophorin C, glycophorin A, and band III. This is consistent with our previous studies\(^2\) showing that the 103B2/9E10 and 105A5 epitopes of CD164 are present on the majority of day-14 CFU-GEMM and BFU-E. Of added interest was the finding that the expression of the 103B2/9E10 epitope was differentially regulated during B-cell development, because the majority of CD34\(^{+}\) B cells in bone marrow expressed the 103B2/9E10 epitope, whereas both mature B cells from peripheral blood and the most immature CD34\(^++\)CD19\(^+\) B-cell precursors were CD164(103B2/9E10 epitope)\(^{lo-}\).

Our recent experiments suggest that ligation of CD34regulated during B-cell development, because the majority of the expression of the 103B2/9E10 epitope was differentially regulated during B-cell development, because the majority of CD34\(^{+}\) B cells in bone marrow expressed the 103B2/9E10 epitope, whereas both mature B cells from peripheral blood and the most immature CD34\(^++\)CD19\(^+\) B-cell precursors were CD164(103B2/9E10 epitope)\(^{lo-}\).

Further to these findings, we have shown by cross-blocking experiments that the binding of the 105A5 MoAb was partially inhibited by prelabeling the cells with the 103B2/9E10 MoAb, but not vice versa. This partial inhibition might be due to the induction, by the 103B2/9E10 antibody, of a conformational change in the CD164 molecule that results in masking the 105A5 epitope. It therefore seems possible that these two epitopes may cooperate or compete with one another in the initial adhesion of CD34\(^+\) cells to stromal cells, with expression of the 105A5 epitope serving to modulate the function of the CD164(103B2/9E10) epitope. Further studies are in progress to fully characterize the epitopes recognized by these different CD164 MoAbs. However, it is possible that the different patterns of expression of the CD164 epitopes on CD34\(^+\) cells reflect differences in glycosylation patterns, carbohydrate modifications, or splice variant expression among different precursor cells within the hematopoietic progenitor cell pool.

One major unanswered question in hematopoiesis involves the mechanisms by which CD34\(^+\) hematopoietic progenitor cells identify particular microenvironmental niches. It is thought that the lodgement of such cells in these niches is required for the maintenance of their quiescent state or for their self-renewal or commitment to particular hematopoietic lineages. It is possible that the sialomucins as a family may act to regulate hematopoietic proliferation or differentiation. Evidence for this comes from both in vitro and in vivo studies. For instance, transfection of the long form of CD34 into M1 cells inhibits their differentiation, whereas transfection of the short isoform has no effect.\(^3\) In other studies, ligation of the CD43 engenders an apoptotic response in more mature cells within the CD34\(^{hi}\)Lin\(^{-}\) subset but not in the more immature stem or severe combined immunodeficiency disease (SCID) repopulating cells.\(^2\)\(^4\)\(^5\) Our recent experiments suggest that ligation of CD164 with both the 103B2/9E10 and 105A5 MoAbs can inhibit the clonogenic growth of CD34\(^{+}\) CD38\(^{lo-}\) cells.\(^7\) An alternative way of determining the potential importance of these mucin-like molecules is to generate knock-out mice lacking the gene of interest or to create transgenic mice overexpressing a particular sialomucin in a specified subset of hematopoietic cells and then to examine their effects on hematopoietic development. Although studies are limited, CD34-null embryonic stem (ES) cells and mice have been generated.\(^3\)\(^9\) In the mutant ES cells, erythroid and myeloid development is delayed during embryoid body formation, but it can be restored by the introduction of either a full-length or cytoplasmically truncated CD34 gene into these cells. In the CD34-null mice, the number of clonogenic progenitor cells is reduced in the yolk sac, fetal liver, adult bone marrow, and adult spleen, and cytokine-induced expansion of bone marrow hematopoietic progenitors ex vivo is inhibited. These studies suggest that cytokines may cooperate with adhesion receptors to induce cell proliferation and/or differentiation by presenting cytokines on the CD34 molecule,\(^5\) by interaction between CD34 and cytokine receptors expressed on the same cell, or by engagement of the CD34 molecule on one cell with its cognate ligand on an opposing cell. Such an adhesion process might precede or be required for interaction with membrane associated growth factors on accessory cells. It will be of interest to see if CD164 possesses similar functions or if signaling via CD164 is required for the regulation of hematopoietic cell proliferation or differentiation.

In the final section of this report, we have identified PAC clones containing genomic fragments of CD164 and have used these to localize the CD164 gene to human chromosome 6q21. These PAC clones have now been fully sequenced and the genomic structure of CD164 determined.\(^1\)\(^2\) These constructs, together with the murine CD164 gene, when cloned, will provide important reagents for identifying elements that regulate CD164 gene expression in hematopoietic progenitor cells and for generating transgenic and knock-out mice for the analysis of the function of CD164 in vivo. In addition, the PAC clones containing CD164 may help to identify other genes on chromosome 6q21 that are related to CD164 or that have similar functions to CD164 in regulating hematopoietic progenitor cell proliferation and differentiation.

ACKNOWLEDGMENT

The authors thank Professor Sir D.J. Weatherall and Professor L. Kanz for their support, Dr D Buck for the AC-133 MoAb, and Heike Hanisch and Doris Schweigert for excellent technical assistance.

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CD164, a Novel Sialomucin on CD34+ and Erythroid Subsets, Is Located on Human Chromosome 6q21

Suzanne M. Watt, Hans-Jörg Bühring, Irene Rappold, James Yi-Hsin Chan, Jane Lee-Prudhoe, Tania Jones, Andrew C.W. Zannettino, Paul J. Simmons, Regis Doyonnas, Denise Sheer and Lisa H. Butler