CD33 is a member of the Ig superfamily that is restricted to cells of the myelomonocytic lineage but whose functions and binding properties are unknown. It shares sequence similarity with sialoadhesin, CD22, and the myelin-associated glycoprotein, which constitute the Sialoadhesin family of sialic acid-dependent cell adhesion molecules. In the present study, we show that CD33 is a fourth member of this family. As a model for sialic acid-dependent binding, human erythrocytes were derivatized with N-acetylmuraminic acid (NeuAc) in different linkages. A recombinant soluble form of CD33, Fs-CD33, bound red blood cells with a specificity similar to that of sialoadhesin, preferring NeuAcα2,3Gal in N- and O-glycans over NeuAcα2,6Gal in N-glycans. Fs-CD33 also bound selectively to the myeloid cell lines HL-60 and U937. However, CD33 was unable to mediate cell binding after transient expression in COS cells, despite high levels of surface expression. Pretreatment of the CD33-transfected cells with sialidase rendered them capable of mediating sialic acid-dependent binding. These results show that CD33 can function as a sialic acid-dependent cell adhesion molecule and that binding can be modulated by endogenous sialoglycoconjugates when CD33 is expressed in a plasma membrane.

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From the Molecular Haemopoiesis Laboratory, Imperial Cancer Research Fund (ICRF), Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Headington, Oxford, UK; and Biochemisches Institut, University of Kiel, Olshausenstraße 40, 24098 Kiel, Germany.


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Address reprint requests to Paul R. Crocker, PhD, ICRF Laboratories, University of Oxford, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU, UK.

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MATERIALS AND METHODS

Materials. Unless otherwise specified, all reagents and chemicals were purchased from Sigma Chemical Co (Poole, UK). Carrierrfree Na235ClO4 was purchased from Amersham International (Slough, UK). Trypsin26,27-Label was from ICN Flow (High Wycombe, UK). Sialidase from Vibrio cholerae was obtained from Calbiochem-Boehringer Corp (La Jolla, CA). Sialidase from Clostridium perfringes and Anthobacter ureafaciens were purchased from Oxford Glycosystems (Abingdon, UK). Protein A Sepharose was purchased from Pharmacia (Milton Keynes, UK). Sialylactose (80% NeuAcα2,3Galβ1,4Glc; 20% NeuAcα2,6Galβ1,4Glc) was from Sigma Chemical Co.

Plasmids. The cDNAs encoding human CD33 in pCDM8,22 human FC-CD33 in pIG, and human FC-NCAM in pIG were the generous gifts of Dr D. L. Simmons (ICRF, Oxford, UK). The FC-CD33 was generated by polymerase chain reaction amplification of the extracellular portion of CD33 cDNA and cloning into Fc expression vector, pIG.25 The Fc-neurial cell adhesion molecule (Fc-NCAM) was generated similarly and consists of the entire extracellular domain (5 IgG-related domains and 2 type III fibronectin repeat-related domains) of the NCAM fused to the Fc fragment of human IgG1.

Cells. All cell lines were provided by the Imperial Cancer Research Fund Cell Production Service (London, UK). COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum (FCS). Other cell lines were cultured in RPMI 1640 medium with 5% or 10% FCS. Human red blood cells (RBCs) were obtained from healthy donors and stored at 4°C in Alsever's solution for up to 2 weeks. To obtain RBCs carrying sialic acid in unique linkages, RBCs were sialidase-treated and then resialylated by incubation with purified sialyltransferases as described previously.25,28

Production of Fc-adhesins and anti-CD33 antisera. COS-1 cells were transfected at 50% to 70% confluence by the diethylaminoethyl-dextran method as above, trypsinized 24 hours later, and replated at 2 × 106/well on 6-well tissue culture dishes (Falcon; Becton Dickinson, Oxnard, CA) in DMEM containing 1% FCS. COS cells treated identically but without plasmid DNA (sham-transfected) were included in each assay. Binding assays were performed for 48 to 72 hours posttransfection. Cells were tested for binding were washed 3 times in PBS, resuspended at 0.25% vol/vol in PBSA plus 5 mmol/L sodium azide for RBCs or 1 × 106/mL for other cell populations, and 1 mL of the cell suspensions was added to the appropriate wells. After 30 minutes at 37°C, nonadherent cells were removed by 3 gentle washes in PBSA. Fab fragments from the polyclonal anti-CD33 antisera, transfected COS cells were preincubated for the last 45 minutes of sialidase treatment with 50 µg/mL of Fab fragments and then washed 3 times in PBS. Fab fragments from a polyclonal rabbit antisialoadhesin antisera were used under identical conditions as a negative control. Immunocytochemical analysis of transfected cells was performed with the polyclonal anti-CD33 antisera at 10 µg/mL IgG using a Vectastain immunoperoxidase ABC kit (Vector Labs, Burlingame, CA). All data shown are representative of at least three separate experiments.

RESULTS

Binding of native and derivatized human RBCs to FC-CD33. Human RBCs can be modified enzymatically to carry sialic acids in unique linkages.28 This provides a useful experimental approach to characterize the specificity of sialic acid-dependent adhesion molecules, as shown recently for sialoadhesin, MAG, and CD22225 and in earlier studies for viruses and bacteria.9,12 In the present experiments, sialidase-treated RBCs were reconstituted to produce RBCs exclusively bearing sialic acid residues in one of the following structures: NeuAcα2,3Galβ1,3GalNAc in O-glycans (3-O), NeuAcα2,3Galβ1,3(4)GlcNAc in N-glycans (3-N), or NeuAcα2,6Galβ1,4GlcNAc in N-glycans (6-N). These oligosaccharides are found commonly on mammalian cells. Previously,28 it was shown that sialoadhesin prefers the 3-0 and 3-N structures and MAG prefers the 3-O structure, whereas CD22 recognizes only the 6-N structure.

To determine whether CD33 could bind human RBCs, we performed solid-phase binding assays with a purified soluble form of human CD33 (Fc-CD33) containing the two extracellular domains fused to the Fc portion of human IgG1. Fc-NCAM was used as a negative control. Native human RBCs attached specifically to immobilized Fc-CD33 (Fig 1A).
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Fig 1. Fc-CD33 binds to human RBCs through recognition of specific sialylated glycans. (A) Fc-CD33 was adsorbed to wells of microtiter plates at the indicated concentrations, and binding assays were performed with human RBCs. $E_{450}$ represents the peroxidase activity of hemoglobin in the bound RBCs. Binding is completely abrogated by sialidase pretreatment of the RBCs. (B) Human RBCs (native) were treated with $V$ cholerae sialidase (asialo), and the sialic acid residues were reconstituted with purified sialyltransferases to give exclusively 3-0, 3-N, or 6-N. Binding assays were performed with Fc-adhesins coated at a concentration of 10 nmol/L. Nonspecific adhesion was determined using Fc-NCAM. (m), Fc-CD33; (U), Fc-NCAM.

Binding was dependent on the concentration of Fc-CD33 used for coating, being maximal at 10 nmol/L, and was sialic acid-dependent because it was completely abolished by pretreatment of the RBCs with sialidase (Fig 1). Adhesion of Fc-CD33 to native RBCs was weaker than that observed in parallel experiments with murine Fc-sialoadhesin and Fc-MAG but was stronger than that of murine Fc-CD22 (data not shown). Similar to sialoadhesin, Fc-CD33 bound derivatized RBCs carrying either the 3-0 or 3-N structures (3-0 > 3-N), although binding to both RBC populations was lower than that with native RBCs (Fig 1B). Binding to RBCs carrying the 6-N structure was negligible. The control protein, Fc-NCAM, did not bind to any of the RBC populations (Fig 1B). These results show that Fc-CD33 shows sialic acid-dependent adhesion with a specificity similar to that of sialoadhesin but distinct from MAG and CD22.

Binding of hematopoietic cell lines to Fc-CD33. To determine whether hematopoietic cell subpopulations carry ligands for Fc-CD33, we performed solid-phase adhesion assays using a panel of human hematopoietic cell lines (Fig 2A). Binding to Fc-CD33 was remarkably selective, because the only cell lines that showed consistently high binding were HL-60 (myelomonocytic-like) and U937 (promonocytic-like). Other hematopoietic cell lines, including those with characteristics of erythroid cells (K562), T cells (Jurkat), B cells (Nalm-6), and monocytes (THP-1), showed little or no binding. The assays were performed at 4°C (Fig 2) and at 37°C (data not shown) with no difference in the profile of adhesion. Binding of HL-60 (Fig 2B) and U937 cells (data not shown) was dependent on the coating concentration of Fc-CD33 and occurred in the complete absence of divalent cations (Fig 2B). Pretreatment of both HL-60 and U937 cells with trypsin led to greatly reduced adhesion to CD33 (data not shown), indicating that the CD33 ligand(s) is glycoprotein in nature.

Sialic acid-dependent adhesion of HL-60 and U937 to Fc-CD33. We next investigated whether binding of HL-60 and U937 to Fc-CD33 is sialic acid-dependent. However, sialidase pretreatment of these cells resulted in variable inhibition of binding in the solid-phase adhesion assay. This was observed even with mixtures of sialidases from $V$ cholerae, C perfringens, and A ureafaciens (data not shown). In previous studies with P-selectin, the sialic acid-dependent adhesion of HL-60 cells was similarly shown to be resistant to sialidase treatment. A possible explanation was that the accessibility of sialidase to the critical sialic acid residues within the P-selectin ligand, PSGL-1, is hindered by the close spacing of oligosaccharides and/or protein conformation. The CD33 ligand(s) might show similar structural characteristics. Alternatively, modifications of sialic acid could result in resistance to sialidase treatment. Partial resialylation of cells during the assays at 37°C could also be a factor.

Sialyllactose was used as a soluble competitive inhibitor of sialic acid-dependent binding (Fig 3). Complete inhibition of binding to Fc-CD33 was observed in the presence of 20 mmol/L sialyllactose for RBCs at 4°C and 37°C and for HL-60 and U937 at 4°C. However, for HL-60 and U937 at 37°C, inhibition by sialyllactose was partial (Fig 3). Binding at either temperature was unaffected in the presence of 20 mmol/L lactose (data not shown). The incomplete inhibition by sialyllactose of HL-60 and U937 binding at 37°C could be because of a temperature-dependent, sialic acid-independent component of adhesion. Alternatively, sialyllactose may be a less effective inhibitor of sialic acid-dependent binding of
HL-60 and U937 cells at 37°C. For instance, CD33 ligands on HL-60 and U937 cells could become clustered at 37°C, leading to more avid binding, whereas RBC ligands may be tethered to the cytoskeleton and unable to cluster.

Adhesion mediated by CD33 expressed on transiently transfected COS cells. Previously, we showed that sialoadhesin, CD22, and MAG bound avidly to native and derivatized human RBCs when expressed transiently in monkey COS-1 cells. Therefore, we used a similar approach to determine whether CD33 could mediate sialic acid-dependent binding when expressed in a plasma membrane. As determined by immunofluorescence, COS cells transiently transfected with CD33 (COS-CD33) consistently failed to bind human RBCs or any of the cell lines despite
CD33 mediates sialic acid-dependent adhesion

Fig 4. Binding of HL-60 cells to COS cells transiently transfected with CD33 cDNA is shown. COS cells were transfected with CD33 cDNA, and binding assays with HL-60 cells were performed after 2 days. Cells were fixed in glutaraldelyde, and the transfected COS cells were identified by immunoperoxidase staining using a rabbit polyclonal antiserum raised to Fc-CD33. The dark, positively labeled cells can be readily distinguished from the unstained, nontransfected cells. (A) No HL-60 binding was observed with untreated CD33-transfected COS cells. (B) HL-60 rosettes were observed when CD33-transfected COS cells were pretreated with V cholereae sialidase. Binding only occurred on the transfected subpopulation of COS cells. (C) Binding of HL-60 cells to sialidase-treated, CD33-transfected COS cells was abolished in the presence of rabbit anti-CD33 Fab fragments at 50 µg/mL. Bar represents 20 µm.

CD33 mediates sialic acid-dependent adhesion. CD33 has only two Ig-like domains, whereas sialoadhesin, CD22, and MAG have 17, 7, and 5 domains, respectively. It is possible that CD33 does not extend sufficiently far out of the COS cell glycocalyx to mediate interactions with ligands on adjacent cells. This could be because of a nonspecific masking effect of the glycocalyx or because of a more specific inhibitory effect. For example, 3-O and 3-N structures in COS cell glycoconjugates could occupy the binding sites of neighboring CD33 molecules and prevent interactions with ligands expressed on apposing cells. To investigate this possibility, COS-CD33 were treated with sialidase to remove sialic acid from 3-O and 3-N structures, before binding assays. This resulted in high levels of HL-60, U937, and RBC binding (Fig 4, Table 1, and data not shown). The adhesion was specific because the HL-60 cells bound only to the CD33-transfected subpopulation (Fig 4B). In addition, binding was abolished because of the presence of Fab fragments from a polyclonal anti-CD33 antiserum (Fig 4C and Table 1) but was unaffected by Fab fragments from an irrelevant polyclonal antiserum (data not shown). Finally, this CD33-mediated adhesion was sialic acid-dependent because sialidase pretreatment of the HL-60 cells prevented binding to the sialidase-treated COS-CD33. Not all positively transfected COS cells bound HL-60 cells (Table 1). This could be because of different levels of CD33 expression in the COS cells and variable efficiency of the sialidase treatments.

Table 1. Binding of HL-60 Cells to CD33-Transfected COS Cells

<table>
<thead>
<tr>
<th>Sialidase Treatment</th>
<th>% Transfected COS Cells Forming Rosettes†</th>
</tr>
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<tbody>
<tr>
<td>COS-CD33 HL-60</td>
<td>Untreated + Anti-CD33 Fab‡</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>− −</td>
<td>0 ND</td>
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<tr>
<td>+ −</td>
<td>34 &lt;1</td>
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<td>+ +</td>
<td>47 3</td>
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<tr>
<td>+ −</td>
<td>0 ND</td>
</tr>
<tr>
<td>+ +</td>
<td>&lt;1 ND</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

* Cells were pretreated with 0.1 U/mL V. cholerea sialidase (+) or with buffer (−) for 2 to 3 hours at 37°C.
† CD33-transfected COS cells were identified by immunoperoxidase labeling with a rabbit polyclonal anti-CD33 antiserum. A positive rosette was defined as a COS cell that had bound more than 5 HL-60 cells. Data represent counts from single wells with at least 200 cells counted per well. Results from three independent experiments are shown.
‡ COS cells were preincubated with Fab fragments from a rabbit polyclonal anti-CD33 antiserum for 45 minutes at 37°C and then washed before the addition of HL-60 cells.

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DISCUSSION

This is the first report showing that CD33 can function as a cell adhesion molecule. The adhesion mediated by CD33 is sialic acid-dependent, with a preference for 3-N and 3-O structures over the 6-N structure. On the basis of sequence similarity and binding properties, CD33 can be considered a fourth member of the Sialoadhesin family. A major difference between CD33 and sialoadhesin, MAG and CD22 is that CD33 is unable to mediate cell adhesion when expressed transiently in untreated COS cells, except after sialidase treatment to remove endogenous ligands. This raises the important question of whether CD33 can normally function as an intercellular adhesion molecule.

The potential for CD33 to mediate adhesion may depend on the relative amounts of CD33 and CD33 ligands expressed on individual hematopoietic cells. Although the nature of the CD33 ligand(s) is unknown, the results obtained with derivatized RBCs show that 3-O and 3-N structures are preferred over the 6-N structure. The repertoire of cell surface carbohydrates is controlled to a large extent by the activities of glycosyltransferases that compete for the various acceptor substrates during biosynthesis. Specific glycosyltransferases can be induced during the terminal stages of myeloid cell development. Some of these could play an important role in the regulation of CD33 ligand expression. As shown for selectin ligands, the nature of the molecules that carry the carbohydrate structures is likely to be important in mediating high-affinity binding. For the CD33 ligands on HL-60 cells, the carrier is probably glycoprotein in nature because binding of Fc-CD33 was lost after trypsin pretreatment.

The results of the present study using hematopoietic cell lines suggest that expression of CD33 ligands depends on the stage of hematopoietic differentiation. In the solid-phase binding assays with Fc-CD33, relatively low levels of ligand were present on KG1 early myeloid precursor-like cells and monocyte-like THP-1 cells, whereas the intermediate-stage myelomonocytic cells, HL-60 and U937, expressed relatively high levels of the CD33 ligand. This implies that CD33 ligands are transiently upregulated during myeloid cell development. Because these myeloid cell lines all express the CD33 molecule, adhesion mediated by CD33 may be regulated at specific stages of myeloid cell differentiation.

Our findings with CD33 bear similarities to recent observations made with the B lymphocyte-restricted molecule, CD22. To date, there have been no convincing experiments showing that CD22 can mediate intercellular adhesion when expressed on the surface of B lymphocytes. CD22 shows strong preference for the 6-N structure whose level is controlled by β-galactoside α2,6 sialyltransferase activity. Expression of this enzyme in B lymphocytes is cell cycle-dependent and is induced after B-cell activation. Most cell-cell binding studies with CD22 have been performed with transfected COS cells that express low levels of the α2,6 sialyltransferase. Similar to CD33, it is possible that sialic acid-dependent binding mediated by CD22 is modulated by interactions with endogenous carbohydrate structures. In support of this possibility, it was recently shown that, when COS cells were cotransfected with both CD22 and α2,6 sialyltransferase cDNAs, cell binding mediated by CD22 was abolished. This could be reversed by pretreating the doubly transfected COS cells with A ureafaciens sialidase that destroys the 6-N structure but not by pretreating with the Newcastle disease virus sialidase that selectively destroys 3-O and 3-N structures.

Thus, for both CD22 and CD33, modulation of binding by endogenous carbohydrate structures may be an important mechanism regulating intercellular adhesion. Could a similar mechanism be important in regulating adhesion mediated by other members of the Sialoadhesin family? Preliminary experiments with a recombinant form of MAG expressed in Chinese hamster ovary cells show evidence for a similar regulatory mechanism (S. Kelm, unpublished observations). However, further studies are required to determine whether the binding properties of MAG, naturally expressed by oligodendrocytes and Schwann cells, can be modulated by endogenous carbohydrates. Sialoadhesin has a similar specificity to CD33 yet is able to mediate specific binding to cells when expressed in COS cells as well as in different macrophage populations. Therefore, it is tempting to speculate that one reason why sialoadhesin has evolved such an extraordinarily large number of Ig-like domains is to extend the NH2-terminal sialic acid binding site away from the macrophage glycocalyx to avoid the potentially inhibitory influence of adjacent carbohydrates. A major challenge for the future will be to understand how regulated glycosylation during hematopoietic differentiation affects the binding and functional properties of CD33 and sialoadhesin, and whether this, in turn, can influence hematopoietic cell growth and differentiation.

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