Neutrophils Express CD52 and Exhibit Complement-Mediated Lysis in the Presence of Alemtuzumab

Running head: PMNs Express CD52 and are Depleted by Alemtuzumab

Lyn R. Ambrose¹, Anne-Sophie Morel¹, Anthony N. Warrens¹, ²

Departments of Immunology¹ and Renal Medicine², Division of Medicine, Imperial College London, Hammersmith Campus, London W12 0NN

Correspondence address:

Lyn Ambrose
Immunology Unit, room 236
Department of Infectious and Tropical Diseases
London School of Hygiene & Tropical Medicine
Keppel St
London WC1E 7HT, UK
Phone: +44 20 7927 2832 e-mail: lynambrose@googlemail.com

Subject heading: Phagocytes, Granulocytes and Myelopoiesis
Abstract

Neutropenia is a recognized adverse event in patients treated with the humanized anti-CD52 monoclonal antibody alemtuzumab. However, as it is widely believed that neutrophils do not express CD52, the etiology of alemtuzumab-associated neutropenia is unclear. We have found that neutrophils express both mRNA coding for CD52 and the protein itself on the cell surface. We confirmed cell-surface expression using three different anti-CD52 antibodies, and note that neutrophils express lower levels of CD52 than lymphocytes and eosinophils. Further, incubation of alemtuzumab with neutrophils results in dose-dependent, complement-mediated lysis in the presence of both heterologous and autologous complement. These data offer an explanation for the etiology of alemtuzumab-associated neutropenia. In a climate of increased use of alemtuzumab in leukemia and other disease states, as well as in transplantation, these data highlight the need for increased vigilance of emerging neutropenia in patients treated with alemtuzumab.
Introduction

Neutropenia is a recognized adverse event in patients treated with alemtuzumab, a humanized monoclonal CD52-specific antibody. A highly lytic antibody, alemtuzumab mediates cytotoxicity by antibody-dependent cell-mediated cytotoxicity and potent activation of human complement. It is approved for use in chronic lymphocytic leukemia (CLL) but is also used in non-Hodgkin’s lymphoma, T cell malignancies, rheumatoid arthritis, vasculitis, scleroderma, eosinophilia, and prevention of graft-versus-host disease and graft rejection in bone marrow, stem cell and solid organ transplantation.

Alemtuzumab administration is sometimes associated with a cytokine-release syndrome, which can include pyrexia, headaches, nausea, urticaria and rigors. Myelotoxicity may result in anemia, thrombocytopenia and neutropenia. In particular, post-alemtuzumab neutropenia occurs in both fludarabine-refractory and treatment-naïve CLL. The etiology of post-alemtuzumab neutropenia, and its associated morbidity and mortality, are poorly understood.

The obvious mechanism for post-alemtuzumab neutropenia would be through neutrophil CD52 expression. However, it is widely believed that neutrophils do not express CD52, unlike T and B lymphocytes, NK cells, monocytes, dendritic cells and male reproductive tract cells. Within the granulocyte population, it has been reported that eosinophils, but not neutrophils, express CD52. We revisited this question and have shown that neutrophils contain CD52 mRNA, express surface CD52, and are susceptible to complement-mediated lysis in the presence of...
alemzumab. The level of CD52 on neutrophils is lower than on eosinophils and T
and B lymphocytes, which could be why it has been difficult to detect.

**Materials and Methods**

**Cell Isolation**

Blood was separated into peripheral blood mononuclear cells (PBMC) and
granulocytes using density-gradient centrifugation over Polymorphprep™ (Axis-
Shield, Norway). RPMI-1640 with 100U/mL penicillin, 100μg/mL streptomycin,
2mM L-glutamine (Invitrogen, UK) and either 2% or 10% human male AB serum
(Biowest, UK) was used for all washes and incubations. Eosinophils were negatively
selected from the granulocytes using CD16-conjugated magnetic beads (Miltenyi,
UK). Permission to use human blood samples was granted by the institutional ethics
review board of Imperial College London and donor informed consent was obtained
in accordance with the Declaration of Helsinki.

**Flow Cytometry**

Cells were stained for 30 minutes at 4°C with saturating concentrations of a specific
monoclonal antibody or an isotype-matched control (Table 1, supplementary data),
acquired on a FACSCalibur™ flow cytometer, and analysed using CellQuest™
software (BD Biosciences).

**RT-PCR**

Total RNA was isolated from cells using an RNeasy mini kit (Qiagen, UK). 15μL
containing 5μg RNA, 0.5μg Oligo-dT primer (Invitrogen) and water was incubated at
65°C for 10 minutes and cooled. To this was added: 1μL dNTP mix (each 10mM) (Promega, UK), 200U M-MLV reverse transcriptase, 7μL 5x buffer, 5μM DTT (Invitrogen), 1μL RNase ribonuclease inhibitor (Promega), and the volume made up to 35μL with water. To perform first-strand cDNA synthesis, this 35μL mixture was incubated at 37°C for 90 minutes. cDNA was amplified in a 25μL reaction mixture containing 0.625U Taq DNA polymerase, reaction buffer (Eppendorf, UK), 0.2mM each dNTP, 0.2μM each primer, and water. The PCR reaction was incubated in a Primus 96 Plus thermocycler (MWG Biotech) with initial denaturation (95°C, 4 minutes), 35 cycles of denaturation, annealing and extension (95°C, 30 seconds; 55°C, 30 seconds; 68°C, 1 minute), and final extension (72°C, 10 minutes).

**Complement-Dependent Cytotoxicity**

Alemtuzumab-induced complement-dependent cytotoxicity was measured using a standard technique: 2x10^3 cells were incubated in Terasaki trays with 1-300μg/mL alemtuzumab for 30 minutes. 5μL of standard rabbit complement (Cedarlane Labs, US) or autologous serum was added and incubated for 60 minutes. Cells were stained with 5μL of vital dye mix (propidium iodide, acridine orange, ink) and read under fluorescence microscopy. The anti-HLA class I mAb (W6/32; Sigma, UK) was the positive control; the negative control was male AB serum (Biowest).
Results and Discussion

In all thirteen individuals studied, we have found that neutrophils contain CD52 mRNA (fig. 3, supplementary data) and express surface CD52 (fig. 1A), albeit at lower levels than lymphocytes (fig. 1B) or eosinophils (fig. 1C). On the basis of relative mean fluorescent intensities, we estimate that neutrophils have 22% the CD52 of lymphocytes. Anti-CD52 titration demonstrated that, at lower mAb concentrations, at which lymphocytes still appear positive (fig. 1Bii), neutrophils appear negative (fig. 1Biii). We speculate that neutrophil CD52 may have hitherto remained undetected due to the use of antibody concentrations that were non-saturating at such low level expression.

We confirmed specificity of the cell-surface protein using three different anti-CD52 antibodies, recognising at least two different epitopes17 (fig. 2, supplementary data). Alemtuzumab binds neutrophil CD52 (fig. 2A, 2B) and induces dose-dependent complement-mediated lysis in the presence of either heterologous or autologous complement (fig. 2Ci and 2Cii, respectively) and the concentration of alemtuzumab observed therapeutically18 certainly exceeds that needed to cause both complement-dependent and antibody-dependent cell-mediated cytotoxicity in vitro18,19.

Alemtuzumab’s ability to activate autologous complement, despite the presence of cell-surface complement regulators, represents one potential mechanism by which alemtuzumab may be therapeutically effective. Incidentally, we did not observe the downregulation of CD16 that is characteristic of neutrophil activation (data not shown), making it unlikely that the adverse effects of alemtuzumab could be...
attributed to it activating neutrophils. In a multicenter trial of alemtuzumab in CLL (n=149)\textsuperscript{14}, 77% of patients developed neutropenia\textsuperscript{20} and 9.5% required G-CSF\textsuperscript{14}. Our data provide one potential mechanism for neutropenia in alemtuzumab-treated patients.

In the only trial to use alemtuzumab with concurrent, prophylactic G-CSF, 64% (9/14) of patients developed grade 3-4 neutropenia\textsuperscript{15}. Four developed late-onset neutropenia (>week 10), which was unresponsive to increased doses of G-CSF but reversed within 3-5 weeks of withdrawing alemtuzumab. It is unclear whether the unresponsiveness to G-CSF was due to alemtuzumab-mediated consumption of mature neutrophils, or alemtuzumab-mediated interference with bone marrow neutrophil development and release. Gilleece and Dexter reported that alemtuzumab treatment does not affect myeloid progenitor cells\textsuperscript{21}, but others reported that Campath-1 (alemtuzumab’s rat precursor) reduces granulocyte macrophage colony-forming cells\textsuperscript{22}. Additionally, it is possible that alemtuzumab-mediated depletion of CD52\textsuperscript{+} neutrophils favors selection of CD52\textsuperscript{-} neutrophil clones, a process known to occur in T cells\textsuperscript{23}, and for other neutrophil surface proteins\textsuperscript{24}.

Neutropenia following alemtuzumab therapy in solid organ transplant recipients is not widely reported, perhaps due to the frequent concomitant use of (neutrophilia-inducing) steroids. Where neutropenia is reported, use of other bone marrow-suppressing agents often renders etiologic conclusions impossible. Considering the current trend towards steroid-free immunosuppression in solid organ transplantation, it is possible that alemtuzumab-associated neutropenia may be unmasked. In the largest series of live donor renal transplant recipients undergoing alemtuzumab and
steroid induction followed by tacrolimus monotherapy (n=205), 15% experienced 40 episodes of neutropenia requiring G-CSF²⁵.

Of course, patients being treated with alemtuzumab are at risk of neutropenia from causes other than the alemtuzumab: the underlying bone marrow disease itself, as a complication of immunosuppression, such as EBV infection, or as an adverse effect of other drugs used.

Our data are of relevance to eosinophil studies which have used CD52 as a marker to positively select eosinophils from a granulocyte population during purification¹⁶. Failure to double-stain the resulting population for CD16 may result in not detecting contaminating (CD52⁺) neutrophils. Additionally, if activated during purification, these neutrophils may express only low levels of CD16, and be mistaken for (CD16⁻) eosinophils.

These data offer an explanation for the etiology of alemtuzumab-associated neutropenia. Our data do not suggest avoiding or discontinuing alemtuzumab, nor do published guidelines.¹ Alemtuzumab is an important and effective treatment and its adverse events are generally predictable, transient and manageable¹. These data simply highlight the need for vigilance for neutropenia following alemtuzumab, especially in patients undergoing medium-to-long term treatment, and in solid organ transplantation regimens avoiding steroids.
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Authorship

Contribution: L.R.A. designed, performed and analyzed the research, and wrote the manuscript. A.S.M contributed to the design and analysis of the molecular work, and donated reagents. A.N.W. contributed to the design and analysis of the research, and to the manuscript.

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Correspondence: Lyn Ambrose, Immunology Unit, room 236, Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel St, London WC1E 7HT. email: lynambrose@googlemail.com.
References


**Figure legends**

**Figure 1. Neutrophils express CD52.**  
A) Purified neutrophils (Ai) and mononuclear cells (Aii) were stained with anti-CD16, anti-CD14 and anti-CD52 mAbs. Surface expression of each of these markers in each of the three populations (R1 in Ai and R2 and R3 in Aii) is shown in Aiii – Axi. Neutrophils were identified on the basis of size and granularity (Ai), and by the expression of a CD16$^{\text{high}}$CD14$^{\text{low}}$ phenotype (Aiii). In this way they were differentiated from monocytes (CD16$^{\text{low}}$CD14$^{\text{high}}$, Aiv) and NK cells (CD16$^{\text{low}}$CD14$^{\text{neg}}$, Av). Neutrophils express CD52 (Avi, Aix). T and B lymphocytes express very high levels of CD52 (Aviii, lower right quadrant), while monocytes (Avii, Ax) and NK cells (Aviii, upper right quadrant) express lower levels.  
B) Unseparated leukocytes were stained with various dilutions of an anti-CD52 mAb (clone HI186). Gating on lymphocytes and neutrophils (R1 and R2 in Bi, respectively), the flow cytograms show that CD52 is expressed at lower levels on neutrophils (Biii) than on lymphocytes (Bii). Similar results were obtained for the anti-CD52 mAb clone YTH34.5 (data not shown). Open histograms represent staining with an anti-CD52 mAb; shaded histograms represent staining with an isotype-matched control mAb of irrelevant specificity.  
C) Using CD16 to differentiate neutrophils (CD16$^{\text{high}}$, Cii) from eosinophils (CD16$^{\text{neg/low}}$, Cvi), we observed that both granulocyte populations express CD52, with significantly lower expression by neutrophils (Cii). Open histograms represent staining with an anti-CD52 mAb; shaded histograms represent staining with an isotype-matched control mAb of irrelevant specificity.
Figure 2: Alemtuzumab binds neutrophil CD52 and induces complement-mediated lysis. A) Unseparated leukocytes were stained with various dilutions of Alemtuzumab. Gating on lymphocytes (R1 in Ai) and neutrophils (R2 in Ai), the flow cytograms show that alemtuzumab binds neutrophil CD52 but requires higher concentrations for detection (Aiii; 3-4, 100-300μg/mL), compared with lymphocyte binding (Aii; 1-4, 1-300μg/mL). B) Gating on a purified neutrophil population (Bi) stained with CD16 (Bii) and alemtuzumab (Biii) confirms the binding of alemtuzumab to neutrophils. C) Neutrophils and mononuclear cells were lysed in a dose-dependent manner in the presence of alemtuzumab with either purified rabbit complement (Ci) or autologous serum (Cii) as the complement source. The anti-HLA class I antibody W6/32 induced cell death in the presence of rabbit complement (Ci), but not autologous serum (Cii), due to the inhibition of autologous complement activation by complement regulators (such as decay accelerating factor and CD59) expressed on PBMCs and neutrophils. In the autologous serum condition, alemtuzumab itself served as the positive control antibody, as its ability to activate human complement is not inhibited by complement regulators. Open histograms represent staining with alemtuzumab; shaded histograms represent staining with an isotype-matched control mAb.
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