Effect of alemtuzumab on neoplastic B cells

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Alemtuzumab (campath-1H) is a humanized antibody directed against the CD52 antigen. CD52 is highly expressed on mature T lymphocytes, monocytes and monocyte-derived dendritic cells as well as on mature B cells and those of B-cell chronic lymphocytic leukemia (B-CLL). Indeed, alemtuzumab has clinical activity in B-CLL in vivo and has been approved by the FDA for the treatment of fludarabine-refractory CLL. The overall response to alemtuzumab is 33% in relapsed or refractory patients whereas it is 87% when the antibody is used as first-line treatment.

Alemtuzumab is also being employed in vivo in non-myeloablative conditioning regimens in bone marrow transplantation (BMT) where it acts through deletion of potentially alloreactive T cells and donor dendritic cells, thus reducing graft-versus-host disease (GVHD). Reduction of GVHD may, however, be accompanied by a reduced graft-versus-leukemia (GVL) effect, leading to increased relapse. In this setting, a direct effect of alemtuzumab on CD52-positive leukemic cells could have a beneficial anti-leukemia effect.

Alemtuzumab is thought to act through the activation of complement and/or antibody-dependent cellular cytotoxicity (ADCC), since the antibody carries the human IgG1 Fc region and mediates complement lysis and ADCC in vitro against T-cells. Furthermore, complement consumption has been demonstrated following administration of alemtuzumab in B-cell non-Hodgkin's lymphoma (B-NHL). In contrast, only limited pro-apoptotic effects of the antibody have been reported, suggesting that immune-mediated activities predominate in the mechanism of action of the antibody. Surprisingly, however, little
work has been done to analyze the capacity of alemtuzumab to effectively carry out complement-mediated lysis (CDC) or ADCC on primary leukemia cells.\textsuperscript{25} Rituximab is also an unconjugated chimeric IgG1 antibody, but directed against the pan-B CD20 molecule. This antibody has been approved for the treatment of B-NHL and has also been used to treat B-CLL.\textsuperscript{25,26} Rituximab and alemtuzumab likely work through similar mechanisms of action.\textsuperscript{25-28} We have previously shown, using AIDS-derived lymphoma cell lines, that alemtuzumab activates complement less well in these cells than does rituximab, even in cell lines which express the same amount of target antigen.\textsuperscript{29} However, in B-CLL, CD20 is usually expressed at much lower levels compared to CD52.\textsuperscript{29,30} We therefore further compared the capacity of both rituximab and alemtuzumab to induce ADCC and CDC in vitro, using the natural neoplastic targets of these two antibodies, namely freshly isolated B-CLL as well as other B-NHL cells.

**Design and Methods**

**Cells**

Heparinized peripheral blood was obtained after informed consent from patients with B-chronic lymphocytic leukemia (B-CLL), B-prolymphocytic leukemia (PLL), mantle cell lymphoma (MCL), marginal zone lymphoma (MZL) and follicular lymphoma (FL) with significant circulating disease (at least 70% neoplastic cells in the mononuclear fraction). All patients were diagnosed by routine immunophenotypic, morphologic and clinical criteria. In all cases double staining with CD19 and slg\(\alpha\), or slg\(\kappa\) was performed; allowing monoclonality to be established and providing the percentage of contaminating normal B cells in the sample which was below 2%. The mononuclear cell fraction was isolated by Ficoll-Hypaque (Seromed, Berlin, Germany) gradient centrifugation and aliquots of cells were frozen in 10% dimethylsulfoxide according to standard procedures.

**Immunofluorescence**

Peripheral blood mononuclear cells (PBMC) from leukemia/lymphoma patients were stained using standard procedures with FITC-conjugated anti-CD52 antibody (rat IgG2a clone YTH34.5, Walter Occhiena, Turin, Italy) or anti-CD20 antibody (clone L27, Becton Dickinson, Mountain View, CA, USA) or FITC-labeled mouse IgG1 control antibody (Becton Dickinson). After washing, stained cells were analyzed in a FACS Calibur instrument (Becton Dickinson) using standard procedures. In order to standardize analyses performed on different days, the negative control was always centered between fluorescence channels 1 and 10, and the gate was set at channel 10.\textsuperscript{31}

**Complement-dependent cytotoxicity (CDC)**

CDC tests were performed as described previously.\textsuperscript{31} Briefly, mononuclear cells were plated in a 100 \(\mu\)L volume at 7×10\(^6\)cells/mL in flat-bottomed 96-well plates in the presence or absence of 10 \(\mu\)g/mL alemtuzumab (a kind gift from Schering, Segrate, Italy) or rituximab (a kind gift from Roche Italia, Monza, Italy) and in the presence of 25% pooled human serum. The cells were incubated for 3 hours at 37°C in 5% CO\(_2\) and then diluted to a final volume of 300 \(\mu\)L in 10% alamar blue solution (Biosource, Camarillo, CA, USA) diluted in complete medium (see below). Incubation was continued overnight at 37°C and the plates were read in a fluorimeter (Cytofluor 23000, Millipore, Bedford, MA, USA) with excitation at 530 nm and emission at 590 nm. Triplicate wells with human serum alone were used as the negative control (0% lysed cells) whereas wells incubated with 0.25% Triton-X100 were used as the positive control (100% lysed cells).\textsuperscript{31} In some experiments, the effect of alemtuzumab in the absence of human serum or in the presence of heat-inactivated serum was analyzed in parallel. Lysis was calculated using the following formula: percentage lysed cells/percentage CD52\(^+\) or CD20\(^+\)cells×100.

**Antibody-dependent cellular cytotoxicity**

Peripheral blood mononuclear cells were purified from patients’ peripheral blood by standard Ficoll-Hypaque (Seromed, Berlin, Germany) gradient centrifugation. The cells were either used immediately or cultured for 48 hours in RPMI1640 medium (Seromed) supplemented with 10% fetal calf serum (FCS, Hyclone Laboratories, Logan, UK), 2mM glutamine (Life Technologies, Paisley, Scotland), 100 U/mL penicillin and 100 \(\mu\)g/mL streptomycin (complete medium), in the presence of 1000 U/mL recombinant human interleukin-2 (rhIL-2; Serono, Rome, Italy).

PBMC (2–3×10\(^6\)) from patients’ samples were labeled by incubation for 2 hours at 37°C in 100–200 \(\mu\)L complete medium containing 1.11 MBq sodium chromate\textsuperscript{31} (Amersham, Little Chalfont, Buckinghamshire, UK). Cells were then washed and incubated for 20 minutes at room temperature with 10 \(\mu\)g/mL alemtuzumab or rituximab, washed again and plated at 10\(^4\) cells/well in round-bottomed 96-well plates. Effector cells were either freshly isolated PBMC or PBMC cultured with IL-2. Increasing numbers of effector cells were added to make effector:target ratios ranging from 2:1 to 63:1, in a final volume of 200 \(\mu\)L. Control wells contained only target cells (to measure spontaneous release) or target cells lysed with 1% sodium dodecyl sulphate (total cpm). The plates were incubated for 6 hours at 37°C in 5% CO\(_2\), after which 100 \(\mu\)L of supernatant were collected from each well and counted in a gamma counter. Percentage lysis was calculated after sub-
stracting the cpm due to spontaneous release (in the absence of effector cells) from all samples using the following formula:

$$\frac{\text{cpm test sample} \times 100}{\text{total cpm}}$$

Results

Lysis of freshly isolated B-CLL through complement activation

We first tested the capacity of alemtuzumab to lyse freshly isolated B-CLL cells in vitro in the presence of human complement, and compared rituximab's capacity in the same setting. The clinical characteristics of the 23 B-CLL patients whose samples were tested are shown in Table 1. Standard CDC assays were performed using 25% pooled human serum as the source of complement and 10 μg/mL of either rituximab or alemtuzumab. Patients' mononuclear cell fractions contained 70–99% leukemic cells. The percentages of cell lysis obtained with either alemtuzumab or rituximab are shown in Figure 1A. Alemtuzumab led to efficient lysis of all samples, cell killing reaching 60–100% (mean 80%). In contrast, and as reported previously, rituximab resulted in very poor lysis in most samples (less than 20% lysis in 19/23 samples and 40–80% lysis in only 4 samples; mean lysis 16%). In order to verify that lysis was due to complement and not to a direct pro-apoptotic effect of alemtuzumab, 16 samples were treated with alemtuzumab in the presence or absence of serum, and cytotoxicity measured as above. The results show that alemtuzumab alone has only a minor effect on cell viability in the absence of complement, with an mean increase in mortality of 8.3% (range 2–14%) in the absence of serum compared to 83% (range 60–100%) for the same samples in the presence of serum. Furthermore the cytotoxicity observed with alemtuzumab in the presence of heat-inactivated serum was comparable to that found in the absence of serum (data not shown). These data demonstrate that the strong lysis induced by the antibody and serum is mostly due to complement activation and that alemtuzumab has little pro-apoptotic activity under the conditions analyzed here.

Lysis of B-CLL by rituximab has been shown previously to be directly proportional to the expression levels of the CD20 antigen in different samples. We therefore compared the levels of expression of CD52 and CD20 in the B-CLL samples analyzed. As shown in Figure 1B, CD52 was highly expressed in all samples (MFI 381–3100, mean 1450) whereas CD20 was generally weakly expressed (MFI 22–750; mean 186).

These data show that alemtuzumab, despite being a poor activator of CDC on lymphoma cell lines which express relatively low amounts of the CD52 antigen (Golay et al. unpublished data), induces CDC very effi-

Table 1. Clinical characteristics of the patients studied.

<table>
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<tr>
<th>Patients</th>
<th>Sex</th>
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<th>No. previous treatments</th>
<th>WBC x10^9/L</th>
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*a all samples were from peripheral blood except those from patients FL1, FL2 and FL3, indicated with *, which were from lymph node biopsies; *staging according to Binet for B-CLL/PLL and according to Ann Arbor for the lymphomas; **none of the patients had been treated previously with alemtuzumab. NA: not available.
ciently in freshly isolated B-CLL, presumably due to the extremely high levels of expression of the antigen in these cells allowing efficient activation of the complement cascade.

**CDC of other neoplastic B cells**

CD52 is highly expressed on normal mature lymphocytes and on T- and B-CLL. Recently CD52 has been shown to be expressed by other neoplastic mature B cells, in particular MCL and HCL. The capacity of alemtuzumab to lyse freshly isolated neoplastic B cells of different subtypes through CDC was therefore determined by testing 1 prolymphocytic leukemia (PLL), 2 HCL, 2 MCL, 1 Burkitt’s lymphoma (BL) and 3 FL. The characteristics of the patients are listed in Table 1. As shown in Figure 2A (black bars), alemtuzumab lysed 70-100% of most samples (mean 88%). As for B-CLL, lysis by alemtuzumab in the absence of serum was negligible in all cases (mean 4.7%) showing that alemtuzumab dose not induce significant apoptosis in B-NHL (data not shown). CDC of the same samples by rituximab was comparable to that of alemtuzumab, with lysis ranging from 55% to 100% (mean 85%) (Figure 2A). Measurement of CD52 expression levels showed that CD52 was highly expressed in all cases (MFI over 520-2600) (Figure 2B). CD20 was generally expressed at lower levels than CD52 (MFI 300-650), except in the two HCL samples which had very high MFI (1500 and 3230) (Figure 2B). We conclude that alemtuzumab efficiently activates complement in all freshly isolated leukemia and lymphoma cells, which express high levels of the CD52 target antigen.

**Lysis of B-CLL through ADCC**

ADCC has been proposed as an important mechanism of action of both alemtuzumab and rituximab. However, we have previously shown that freshly isolated leukemic cells, unlike cell lines, are poorly lysed in vitro through ADCC using rituximab and peripheral blood NK cells as effector cells. We therefore wanted to investigate whether alemtuzumab induces more efficient ADCC of leukemic B cells in vitro than does rituximab in the same conditions. Standard ADCC assays were performed at different effector:target (E:T)
ratios using PBMC (containing an average 11% of CD56/CD16+ NK cells) as the effector cells and a series of leukemic samples, including 6 B-CLL, 1 PLL, 2 MCL, 2 FL and 1 MZL. All samples were tested in the presence or absence of either rituximab or alemtuzumab. The results of the dose response curves for 4 out of 7 B-CLL/PLL samples tested are shown in Figure 3 (left panels). The other 3 B-CLL gave comparable results (data not shown). None of the samples was efficiently lysed by freshly isolated PBMC in the absence of antibody (lysis 0–10%; mean 3.2% at the highest E:T ratio) (Figure 3A-D, open circles). As previously reported, rituximab did not induce significant lysis (mean 4.8% at the highest E:T ratio) (Figure 3A-D, closed circles, data not shown). Alemtuzumab also mediated very low lysis, reaching a mean of 10% (i.e. 7% above background) for all 7 B-CLL and PLL cases analyzed (Figure 3A-D, closed squares and data not shown). Similar results were obtained with the other neoplastic B cells tested (MCL, FL, MZL) (Figure 4). Lysis by both alemtuzumab and rituximab was usually less than 10% in all cases (Figure 4, left hand panels A–E). The only exceptions were the two FL samples analyzed which showed 30–44% lysis above background in the presence of alemtuzumab at the highest E:T ratio (Figure 4, panels C+D, closed squares). We have previously shown that rituximab-mediated ADCC of freshly isolated leukemic samples is greatly enhanced by pre-activation of the effector cells by short-term culture in interleukin-2. 38 We therefore compared the ADCC mediated by IL-2-activated NK cells using either alemtuzumab or rituximab. The full dose response curves are shown in Figure 3 E–H and Figure 4 F–J (right hand panels). The data clearly confirm that IL-2 increases background lysis in the absence of antibody in most cases as well as specific ADCC with either alemtuzumab...
tuzumab or rituximab. Indeed significant ADCC was obtained at much lower effector:target ratios using IL-2-activated PBMC (E:T ratios 2–7), than when using freshly isolated effector cells (E:T ratios 21–63), and specific lysis was higher at several effector:target ratios compared to ADCC in the absence of IL-2 culture (Figures 3 and 4). We conclude that the ADCC of most freshly isolated leukemic samples with alemtuzumab and PBMC is weak, and in particular that of B-CLL/PLL, despite the very high levels of CD52 antigen expressed on the target cells. However, this lysis, similarly to that previously observed with rituximab, can be greatly enhanced by activation of effector cells with IL-2 in vitro.

Discussion

In this study we investigated the capacity of alemtuzumab to lyse freshly isolated neoplastic B cells through either CDC or ADCC in vitro and compared this activity with that of rituximab. We show that alemtuzumab lysed B-CLL cells through CDC very efficiently, reaching a mean lysis of 80%. Alemtuzumab alone had little cytotoxic activity in the absence of complement suggesting that it has limited pro-apoptotic activity, in agreement with a recent report.26 Rituximab on the other hand activates complement poorly in B-CLL, with the mean lysis being 16% in the same B-CLL samples.27 The different CDC levels obtained with alemtuzumab compared to rituximab presumably reflect the amount of target antigens expressed by B-CLL cells, since CD20 is weakly expressed in most samples (MFI below 300), whereas CD52 is extremely strongly expressed (MFI 380–3100) and CDC has previously been shown to correlate with target antigen levels.31,32 In other neoplastic B-cell samples on the contrary, both alemtuzumab and rituximab were very effective, killing a mean of 88% and 85% of the cells, respectively, in the presence of human complement. This amount of lysis corresponded to an expression level of both target antigens above MFI 400. These results are interesting in view of our previous observation that AIDS–derived cell lines are resistant to lysis by alemtuzumab, but not rituximab; this resistance was shown even by cell lines expressing equal amounts of CD20 and CD52.25 Indeed we also tested 5 non-AIDS–derived follicular and Burkitt’s lymphoma cell lines, all of which showed the same resistance to CDC by alemtuzumab (mean lysis 7%) (Golay et al., unpublished data). In all cases CD52, although expressed on at least 90% of cells, was relatively dim (MFI below 250). Thus adaptation of lymphoma cells during long-term in vitro culture appears to result in a decrease in CD52 expression, and a consequent resistance to in vitro lysis with alemtuzumab and complement.

In contrast, we show here that most freshly isolated leukemia/lymphoma cells derived from mature B cells (CLL, PLL, MCL, FL) express high levels of CD52 and are efficient targets for alemtuzumab and complement. This observation is important in view of the increasing clinical use of alemtuzumab as an immunosuppressive agent in the context of non-myeloablative conditioning for bone marrow transplantation.15–17 This procedure has been demonstrated to reduce transplantation–related mortality significantly in allogeneic transplantation. In this clinical setting, the capacity of alemtuzumab to deplete normal T and B cells from host and donor origin allows good donor cell engraftment and low GVHD.15,16,20 Furthermore in all neoplasias expressing high levels of CD52, alemtuzumab may also, in principle, have an anti-leukemia effect through complement activation and ADCC; thus counteracting the reported reduction of the allogeneic T-cell– or NK-mediated GVL effect in this setting.15,17,24 A mechanism of action that relies on complement may be particularly relevant in this clinical context, since alemtuzumab may also deplete the NK cells which may be required for ADCC and/or direct tumor cell killing. It is, however, worth remembering that the standard dose of alemtuzumab in the bone marrow transplantation setting is much lower than that used to treat B-CLL (20 mg/day for 5 consecutive days compared to 30 mg/3 times/week for 12 weeks, respectively) with consequent lower maximal serum levels (10 µg/mL versus 20–25 µg/mL, respectively).25,26,28,29 Potentially reduced anti-tumor efficacy. Our results suggest that complement activation is likely to be an important mechanism of action of alemtuzumab in vivo, as it is for rituximab.23,25,26 Arguments in favor of ADCC as the major mechanism of action of alemtuzumab were presented in the past following a study of different anti–CD52 antibody isotypes in vivo.22 This latter study, however, used only rat IgM, IgG2a and IgG2b isotypes, but not the humanized alemtuzumab itself.30 Here we show that alemtuzumab, like rituximab, gave low percentages of lysis through ADCC of freshly isolated leukemic samples with a mean of 7% for B-CLL/PLL samples, and 13% for all leukemia/lymphoma samples tested. Thus lysis with alemtuzumab remained low despite the very high expression level of CD52 antigen on leukemic targets, estimated to be a mean of 37,000 molecules per cell in B-CLL compared to 8,000 for CD20.41 These results are not easily reconciled with the hypothesis that ADCC is a major mechanism of action of alemtuzumab. In vitro however the resistance of leukemic cells to ADCC can be at least in part overcome by culturing PBMC in IL-2 for 48 hours. Thus using activated NK cells, specific lysis was either increased and/or was obtained at lower effector:target ratios. We and others have previously shown that short-term culture in IL-2 does not increase the percentage of NK cells.42,43
The exact mechanism involved in IL-2-mediated NK-cell activation still needs to be fully determined, but is likely to involve induction or activation of natural cytotoxicity receptors, in particular NKp44 and NKG2D. Whether the events that take place following administration of therapeutic monoclonal antibodies to patients, such as complement activation and release of cytokines, can lead to similar NK-cell activation in vivo is unknown at present. Furthermore it is possible that, in vivo deposition of complement fragments on target cell membranes permits improved ADCC through interaction with complement fragment receptors such as CR3 on NK cells.

The results presented demonstrate that lymphoma and leukemia samples derived from the mature B-cell compartment are good targets for the cytolytic activity of alemtuzumab and human complement. This suggests that the use of alemtuzumab in the non-myeloablative regimen for allogeneic stem cell transplantation may be particularly beneficial in this category of neoplasias, in which the antibody may also provide some direct GVL effect. In addition the data presented offer a biological rationale for the combined use of IL-2 and unconjugated monoclonal antibodies in clinical practice. Recent data suggest that the combination of low dose IL-2 and rituximab or trastuzumab is feasible and leads to increased NK-cell-dependent cytotoxic activity in vitro. The clinical benefit does, however, remain to be clearly demonstrated.

JG performed some experiments, supervised the experimental work, wrote the manuscript and participated in the fund raising. MM performed most experiments and prepared the artwork. AR and MI conceived the project, participated in discussions on the project, critically read the manuscript and participated in fund raising. AR supervised the clinical aspects of the project. The authors reported no potential conflicts of interest.

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