CD20 monoclonal antibody therapy for B-cell lymphoma

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Analysis of CD20-dependent cellular cytotoxicity by G-CSF-stimulated neutrophils

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Abstract

Rituximab, a chimeric CD20 monoclonal antibody (mAb), is widely used in the treatment of patients with low-grade non-Hodgkin’s lymphoma. Possible anti-tumor mechanisms involve complement-mediated lysis and/or antibody-dependent cellular cytotoxicity (ADCC). Because G-CSF greatly enhances the cytotoxicity of neutrophils (PMN) in ADCC, the clinical efficacy of rituximab might be enhanced by the addition of G-CSF. Therefore, we investigated the neutrophil-mediated CD20-dependent cellular cytotoxicity in B-cell lines. In contrast to previous studies by others, we found that G-CSF-primed PMN are capable of functioning as effector cells in CD20-dependent cellular cytotoxicity. However, HLA class II mAbs were far more effective. The differences between HLA class II- and CD20-mediated PMN-ADCC were not due to: 1) the use of chimeric (hIgG1) mAbs versus mlgG2a mAbs; 2) HLA class II-induced apoptosis as an ‘ADCC-sensitizing’ mechanism; 3) CD20-induced inhibition of ADCC; 4) inferior membrane mobility of CD20. Analysis of Fcy receptor (FcγR) involvement showed that although CD20-induced ADCC was mediated mainly via FcγRI, for optimal lysis FcγRI and FcγRII were both required. In contrast, in HLA class II-dependent ADCC both FcγRI and II were capable of independently inducing maximum lysis. The exact mechanism underlying the difference in quality of the FcγR-binding and activation by chimeric CD20 mAbs as compared to HLA class II mAbs, remains to be elucidated.
Introduction

Rituximab (IDEC-C2B8), a chimeric CD20 monoclonal antibody (mAb), is nowadays widely used in the treatment of patients with relapsed low-grade B-cell lymphoma.\textsuperscript{13} It is composed of human IgG1 and kappa constant regions and variable antigen recognition sites from the murine CD20 mAb IDEC-2B8.\textsuperscript{4} Rituximab is directed against the B-cell specific antigen CD20, a nonglycosylated 33 to 37 kD phosphoprotein, expressed on more than 95% of normal and malignant B-cells. During B-cell differentiation, the CD20 antigen is present from the pre-B cell stage onwards and absent on plasma cells. Importantly, CD20 is not expressed on other hematologic cells or nonlymphoid cells. Several studies suggest that CD20 acts as a calcium channel and plays a role in B-cell activation, proliferation and differentiation (reviewed by Tedder and Engel\textsuperscript{5}).

In vitro, rituximab induces apoptosis\textsuperscript{6,7}, complement-dependent cytotoxicity (CDC)\textsuperscript{8,9} and antibody-dependent cellular cytotoxicity (ADCC)\textsuperscript{4,8} in malignant B-cell lines. The efficacy of rituximab in ADCC has been shown with mononuclear cells (MNC) as effector cells.\textsuperscript{4,8} Freshly isolated neutrophils (PMN) can also function as effector cells in ADCC.\textsuperscript{10-12} However, the cytotoxicity of PMN in ADCC can be greatly enhanced by exposure to several cytokines, such as Interferon-\gamma (IFN-\gamma) or granulocyte colony-stimulating factor (G-CSF).\textsuperscript{10,12-15} PMN from healthy donors express FcyRII (CD32) and FcyRIII (CD16). FcyRII is the main cytotoxic FcyR of unstimulated PMN.\textsuperscript{11,12} After stimulation with IFN-\gamma (in vivo and in vitro) or G-CSF (in vivo), PMN additionally express FcyRI (CD64), the only FcyR that can bind monomeric IgG with high affinity.\textsuperscript{16,17} FcyRI has been reported to be the main cytotoxic trigger-molecule involved in ADCC with G-CSF-primed PMN.\textsuperscript{10,12,13,17}

Theoretically, the clinical anti-tumor efficacy of monoclonal antibodies can be enhanced by increasing the cytotoxic capacity of effector cells in ADCC. Thus, adding G-CSF to rituximab treatment could enhance the efficacy of the antibody, by increasing the cytotoxic activity of PMN in CD20-dependent killing of B-cells. However, in previous studies the capacity of CD20 mAbs to induce neutrophil-mediated ADCC in malignant B-cell lines has been questioned.\textsuperscript{12} Rather unexpectedly, HLA class II mAbs were found to be the only mAbs capable of mediating PMN-ADCC of B-cells.\textsuperscript{10,12} No satisfactory explanation for these observations has been offered.

In the present study we performed a detailed analysis of the PMN-mediated CD20-dependent cellular cytotoxicity in B-cell lines and compared it with HLA class II-mediated cytotoxicity.
Materials and Methods

Monoclonal antibodies (mAbs) and reagents

Chimeric CD20 mAbs (IDEC-C2B8, rituximab) were obtained from Roche Nederland B.V. (Mijdrecht, The Netherlands). Murine CD20 mAbs (NKI-B20/2a, mlgG2a) were kindly provided by Dr. T. Valerius (Friedrich-Alexander-Universität, Erlangen-Nürnberg, Germany). F(ab')₂ fragments of HLA class II antibodies were produced by pepsin degradation as described previously. Control antibody mlgG2a was from the CLB (Amsterdam, The Netherlands). Control antibody hlgG1 was isolated from pooled human serum in our laboratory (CLB, Amsterdam, The Netherlands).

Goat anti-human IgG (Fcγ fragment specific) antibodies and F(ab')₂ fragments of goat anti-mouse IgG (H+L) antibodies (both from Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) were used as crosslinking antibodies (referred to as GAH and GAM respectively).

CD64 mAbs (197 to FcγRI; whole antibody, mlgG2a) were from Medarex (Annandale, NJ, USA) and CD16 mAbs (CLB-FcRgran1 to FcγRIII) were from the CLB (Amsterdam, The Netherlands). CD32 mAbs (IV.3 to FcγRII) were purified from hybridoma culture supernatant in our own laboratory. Fab' fragments of IV.3 and F(ab')₂ fragments of CLB-FcRgran1 were produced in our own laboratory.

FITC-labeled CD3 mAbs (Leu-4; mlgG1), FITC-labeled CD7 mAbs (Leu-9; mlgG2a), PE-labeled CD56 mAbs (Leu-19; mlgG1) and PE-labeled control antibodies mlgG1 and mlgG2a were from Becton Dickinson (San Jose, CA, USA). FITC-labeled CD66b mAbs (B13-29; mlgG1) and FITC-labeled control antibodies mlgG1 and mlgG2a were from the CLB (Amsterdam, The Netherlands). The direct labeled antibodies were used to analyze the purity of the effector cell populations by flow cytometry (FACS-scan, Becton Dickinson, San Jose, CA, USA).

Texas Red Phalloidin (Molecular Probes, Eugene, Oregon, USA), FITC-labeled goat F(ab')₂ anti-mouse IgG + IgM (H+L) antibodies (Kallestad, South Austin, TX, USA) and FITC-labeled goat F(ab')₂ anti-human IgG antibodies (Caltag Laboratories, Burlingame, CA, USA) (further referred to as GAM-FITC and GAH-FITC respectively) were used to stain cytospin preparations. Annexin V and propidium iodide were from Bender Medysystems (Vienna, Austria).

Cell lines

The Burkitt-lymphoma cell lines Raji and Ramos were obtained from the ATCC. Cell lines were cultured in Iscove's Modified Dulbecco Medium (IMDM; Bio Wittaker, Brussel, Belgium), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Gibco Life Technologies, Paisley, Scotland), 100 U/mL penicillin, 100 mg/mL streptomycin and L-glutamin (Sigma Chemical Co., St. Louis, MO, USA).
Isolation of neutrophils, mononuclear cells and monocytes

After informed consent, blood was obtained either from healthy volunteers or from patients with low-grade NHL treated in a phase I/II study to evaluate the safety and efficacy of the combination of chimeric CD20 monoclonal antibodies (IDEC-C2B8, rituximab) and G-CSF. In this study, patients were treated with 375 mg/m² rituximab weekly for four weeks. G-CSF (Neupogen; Amgen, Thousand Oaks, Ca, USA; 5 μg/kg/day subcutaneously) was administered on three consecutive days, starting two days before each infusion. Venous blood samples (EDTA-anticoagulated) were obtained after the three injections of G-CSF, just before starting the antibody-infusion.

Neutrophils and mononuclear cells were isolated as previously described. Briefly, 5-10 mL of EDTA-anticoagulated blood was centrifuged over a continuous Ficoll gradient (Ficopaque, Pharmacia Biotech, Uppsala, Sweden). After centrifugation, mononuclear cells were harvested from the interphase and the pellet fraction was treated with ice-cold isotonic NH₄Cl solution (155 mmol/L NH₄Cl, 10 mmol/L KHCO₃, 0.1 mmol/L EDTA, pH 7.4) to lyse erythrocytes. The granulocytes and the mononuclear cells were washed twice (the first time in phosphate-buffered saline (PBS), the second time in culture medium) and cells were resuspended in culture medium at a concentration of 10⁷ cells/mL.

Purity of the granulocytes, as determined by cytopsin preparations, was greater than 98%, with >95 % neutrophils. The percentage of natural killer (NK-) cells in the granulocyte fraction was <0.5% as determined by flow cytometry (FACS-scan, Becton Dickinson, San Jose, CA, USA) using FITC-labeled CD7 and PE-labeled CD56 antibodies to detect NK-cells (CD7+/CD56+). Viability of the granulocytes, tested by trypan blue staining, always exceeded 98%. Monocytes were isolated from pooled buffy coats from healthy volunteer blood donors by means of density centrifugation followed by elutriation. Monocytes were stored in plastic ampoules in liquid nitrogen. After thawing, viability of the cells was >95%, as shown by trypan blue exclusion.

ADCC assays

ADCC assays were performed as described. In brief, target cells were labeled with ⁵¹Cr (100 mCi per 10⁶ cells) for 1 hour at 37 °C. Cells were washed three times and resuspended in culture medium at a concentration of 0.2 x 10⁶ cells/mL. Cells were then sensitized with antibodies (final concentration 5 μg/mL). Neutrophils and target cells were added to 96-wells flat bottom microtiter plates (NUNC Brand Products, Denmark) in an effector-target (E:T) ratio of 40:1 or 100:1 and adjusted to a final volume of 200 μL. The plates were centrifuged at 200 g for 1 min and incubated for four hours at 37 °C and 5% CO₂. In some experiments, blocking antibodies to Fcγ receptors (final concentration 10 μg/mL) were added to the neutrophils, 45 minutes before the addition of target cells. After four hours,


\( ^{51}\text{Cr} \) release was measured in triplicate and expressed as counts per minute (cpm) (Cobra, Canberra Packard Instrument Company, Meriden, CT, USA). Percentage of specific lysis was calculated using the following formula: \( \% \) specific lysis = \( \frac{\text{experimental cpm-spontaneous cpm}}{\text{maximal cpm-spontaneous cpm}} \) x 100.

The maximal \( ^{51}\text{Cr} \) release was determined by adding saponin (5% m/v, 100 \( \mu \text{L} \)) to target cells; the spontaneous \( ^{51}\text{Cr} \) release was determined by measuring \( ^{51}\text{Cr} \) release from unsensitized target cells in the absence of effector cells. Spontaneous release was always <10\% of the maximum release for Raji cells and <30\% of the maximal release for Ramos. Controls of antibody-sensitized target cells (without effector cells) showed low levels of cytotoxicity (<2\% in Raji and <10\% in Ramos). Controls of target cells and effector cells in the absence of antibody showed <2\% cytotoxicity by HD PMN and G-CSF PMN. Controls in the presence of mononuclear cells (MNC) were somewhat higher (see fig. 2).

For analysis of the effects of FcyR-blocking antibodies on the specific lysis, the following formula was used: \( \% \) inhibition = \( \frac{\% \text{ specific lysis in the absence of blocking antibody - } \% \text{ specific lysis in the presence of blocking antibody}}{\% \text{ specific lysis in the absence of blocking antibody}} \) x 100

Negative values are reported as percentage of stimulation in the presence of the blocking antibody.

Induction and detection of apoptosis

B-cells (Ramos and Raji) (0.2 \( \times 10^6/\text{mL} \)) were either not stimulated or incubated with the indicated antibodies (final concentration 5 \( \mu \text{g/mL} \) unless indicated otherwise) with or without crosslinking antibodies (final concentration 50 \( \mu \text{g/mL} \)) for different periods of time (see under Results). Apoptotic cells were detected by measuring phosphatidyl serine (PS) exposure on the membrane as described previously.\(^{24}\) In brief, cells were harvested and washed twice in ice cold Hepes buffer (20 mM Hepes, 132 mM NaCl, 6 mM KCl, 1 mM MgSO\(_4\), 1.2 mM K\(_2\)HPO\(_4\), pH 7.4) supplemented with 2.5 mM Ca++, 1 mg/mL glucose and 2.5\% (v/v) albumin. Cells were then incubated with saturating amounts of FITC-labeled annexin V (diluted in HEPES buffer) for 30 min and washed again in HEPES buffer. Just before analysis of the samples by flow cytometry (FACS-scan, Becton Dickinson, San Jose, CA, USA), propidium iodide (PI) was added to the cells (final concentration 5 \( \mu \text{g/mL} \)) to distinguish necrotic cells (annexin V/PI\(^+\)) from apoptotic cells (annexin V\(^-\)/PI\(^-\) (early apoptotic cells) and annexin V\(^-\)/PI\(^+\) (late apoptotic cells)).

Confocal microscopy

B-cells (Raji) were incubated with HLA class II mAbs or chimeric CD20 mAbs (5 \( \mu \text{g/mL} \)), in the presence or absence of crosslinking antibodies (GAM or GAH, 50 \( \mu \text{g/mL} \)) for 4 hours.
Cytospins were prepared and slides were fixed with 2 % paraformaldehyde (PFA; w/v) and permeabilized with 0.5 % Triton X-100. Slides were stained with Texas Red Phalloidin (diluted 1/200) to detect F-actin and GAM-FITC (diluted 1/10) or GAH-FITC (diluted 1/16) to detect HLA class II mAbs and CD20 mAbs respectively. Slides were evaluated by confocal microscopy (LSM510, Carl Zeiss, Inc.)

Statistics
Statistical analyses were performed using the SPSS statistical package (SPSS for Windows, version 8.0). All data are represented as mean ± SEM. Differences between groups were evaluated using the Wilcoxon Signed Ranks test. A P-value ≤ 0.05 was considered significant.

Results
Different cytotoxic capacity of neutrophils in CD20-dependent and HLA class II-dependent cellular cytotoxicity

Freshly isolated PMN from healthy donors (HD PMN) were unable to kill Raji cells sensitized with either chimeric CD20 mAbs or HLA class II mAbs (fig. 1). In contrast, PMN obtained after in vivo G-CSF-stimulation (further referred to as G-CSF PMN) could kill CD20-coated Raji cells: specific lysis was 10 ± 2 % at an effector-target (E:T) ratio of 40:1 and 21 ± 5 %

Fig. 1 G-CSF stimulated neutrophils mediate ADCC of CD20-sensitized Raji cells
Lysis of Raji cells by PMN after sensitization of the cells with chimeric CD20 mAbs (hlgG1), HLA class II mAbs (L243, mlgG2a) or murine IgG2a CD20 mAbs (NKI-B20/2a). Values depicted are mean ± SEM of indicated number of experiments. Controls of target cells without antibody or without effector cells were both < 2%. Abbreviations: PMN= neutrophils; HD= healthy donor neutrophils; E:T= effector-target ratio; G-CSF= in vivo G-CSF-stimulated neutrophils.
at an E:T ratio of 100:1 (fig. 1). In the presence of HLA-class II mAb L243 however, lysis induced by G-CSF PMN was considerably higher: 54 ± 6% (E:T ratio of 40:1) and 77 ± 5% (E:T ratio 100:1). In the absence of effector cells, no lysis was observed with either antibody (data not shown). To evaluate whether the difference between HLA class II (mlgG2a)- and CD20 (hlgG1)-dependent ADCC was due to the difference in isotype between these antibodies, murine IgG2a CD20 mAbs (NKI-B20/2a) were tested in PMN-mediated ADCC. NKI-B20/2a mAbs did not induce ADCC (fig. 1), indicating that the difference in isotype is not the explanation for the large difference in observed lysis.

Crosslinking of CD20 with goat-anti-human IgG antibodies (50 μg/mL) did not induce lysis of Raji cells, nor did crosslinking of HLA class II mAbs with goat-anti-mouse IgG antibodies. Even after 24 hours of incubation, the percentage of specific lysis was below 5% (not shown). These data indicate that the observed PMN-mediated cytotoxicity was not just induced by crosslinking of the antibodies by PMN-FcyR, but that a biologic response of the PMN is required to induce cell lysis.

The capacity of neutrophils to mediate CD20-dependent ADCC was compared to that of MNC. As shown in fig. 2, MNC killed CD20-sensitized Raji cells as effective as HLA class II-sensitized Raji cells. Thus, G-CSF-stimulated neutrophils were more effective than MNC in HLA class II-dependent ADCC, and less effective than MNC in CD20-dependent ADCC. Cytotoxicity by MNC induced by either antibody could be inhibited by blocking FcyRIII, but not by blocking FcyRI. Because purified monocytes did not induce lysis of CD20- or HLA class II-sensitized Raji cells (data not shown), we conclude that NK cells are the main effector

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**Fig. 2** No difference in MNC-induced lysis of CD20- or HLA class II-sensitized Raji cells

ADCC of Raji cells mediated by freshly isolated MNC (E:T ratios of 40:1 and 100:1) in the presence of chimeric CD20 mAbs (■), HLA class II mAbs (L243) (□) or without sensitizing mAbs ( ). Values depicted are mean ± SEM of indicated number of experiments. Abbreviations: MNC= mononuclear cells; HD= healthy donor; E:T= effector-target ratio.
cells within the mononuclear cell fraction. Similar results on both PMN- and MNC-mediated ADCC were obtained with the Burkitt lymphoma cell line Ramos (data not shown), indicating that the CD20-induced PMN-ADCC was not restricted to one single B-cell line.

HLA class II-induced apoptosis does not explain the high HLA class II-mediated cellular cytotoxicity by neutrophils

It is known that ligation of HLA class II (i.e. in the absence of effector cells) induces apoptosis in B cells, whereas CD20 mAbs only induce apoptosis after crosslinking. In Raji cells, ligation of HLA class II induces apoptosis in ~40% of the cells after 4 hours of incubation (as measured by annexin V staining; fig. 3A) and in ~70% of the cells after 24 hours of incubation (fig. 3B). We tested the possibility that ligation of HLA class II initiates apoptosis, thereby rendering the B cells more susceptible to PMN-mediated cytotoxicity.

In these experiments, Raji cells were incubated simultaneously with F(ab')\text{2} fragments of HLA class II mAb L243 and chimeric CD20 mAbs. Binding of L243-F(ab')\text{2} fragments did

Fig. 3 A+B) F(ab')\text{2} fragments of HLA class II mAbs induce apoptosis in Raji cells

Raji cells were incubated with chimeric CD20 mAbs (5 \(\mu\)g/mL), HLA class II mAb L243 (5 \(\mu\)g/mL) with or without crosslinking antibodies (goat anti-human mAbs (GAH) or goat anti-mouse mAbs (GAM), 50 \(\mu\)g/mL), or with F(ab')\text{2} fragments of L243 (10 \(\mu\)g/mL) for 4 hours (A) or 24 hours (B). Apoptosis was measured by annexin V staining as described in Materials and Methods. Data are corrected for the spontaneous amount of apoptosis. Values are mean ± SEM of at least 3 experiments (nd = not done). C) Ligation of HLA class II does not increase susceptibility of B cells to neutrophil-mediated cytotoxicity

ADCC of Raji cells mediated by G-CSF primed neutrophils (E:T ratio 100:1) in the presence of CD20 mAbs (5 \(\mu\)g/mL), HLA class II mAb L243 (5 \(\mu\)g/mL), F(ab')\text{2} fragments of L243 alone (10 \(\mu\)g/mL) or the combination of CD20 mAbs (5 \(\mu\)g/mL) and F(ab')\text{2} fragments of L243 (10 \(\mu\)g/mL).
not interfere with the binding of CD20 mAbs (data not shown). Furthermore, the F(ab')\(_2\) fragments of L243 were found to induce apoptosis in Raji cells to a similar extent as observed with the complete HLA class II mAb L243 (fig. 3A+B).

As shown in fig. 3C, the L243-F(ab')\(_2\) fragments alone did not induce PMN-mediated lysis. L243-F(ab')\(_2\) fragments did not enhance CD20-mediated apoptosis. Thus, HLA class II-induced apoptosis does not explain the difference between HLA class II- and CD20-mediated cellular cytotoxicity by neutrophils. Furthermore, chimeric CD20 mAbs did not inhibit HLA class II-induced cellular cytotoxicity by neutrophils nor HLA class II-induced apoptosis (not shown).

Analysis of FcγR's involved in lysis of HLA class II- or CD20-sensitized B-cells

Although FcγRI has been reported to be the main molecule involved in cytotoxicity of in vivo G-CSF stimulated PMN\(^{12,17}\), FcγRII is also capable to function as a trigger molecule of G-CSF PMN.\(^{10-12}\)

To analyze the relative contribution of FcγR on CD20 and HLA class II-mediated lysis, the effect of blocking mAbs to FcγR on ADCC was investigated. Lysis of CD20-coated Raji cells by G-CSF PMN could be inhibited most efficiently by anti-FcγRI (69 ± 8% inhibition of specific lysis versus 33 ± 7% inhibition by anti-FcγRII) (fig. 4). Lysis was completely blocked if anti-FcγRI and anti-FcγRII were combined. These results indicate that although both FcγR are involved in CD20-dependent ADCC, FcγRI seems to be the main cytotoxic trigger molecule in CD20-dependent ADCC.

Fig. 4 Different FcγR involved in CD20- and HLA class II-mediated ADCC

Neutrophil-mediated ADCC-experiments were performed in the presence of blocking antibodies to FcγR. Antibodies against FcγRI, FcγRII and FcγRIII were tested either alone (10 μg/mL) or in different combinations (10 μg/mL each). Values are mean ± SEM of indicated number of experiments.

In contrast, HLA class II-induced cytotoxicity was not inhibited by mAbs to FcγRI or FcγRII alone (or in combination with anti-FcγRIII), whereas HLA class II-induced ADCC was completely inhibited by anti-FcγRI and anti-FcγRII together (fig. 4). These results suggest,
that in HLA class II-dependent cytotoxicity both FcγRI and FcγRII independently have the capacity to induce maximum lysis of target cells. Blocking of FcγRIII stimulated both CD20- and HLA class II-mediated cytotoxicity, indicating that FcγRIII is not involved in ADCC as effector FcγR. This is consistent with previous studies on the cytotoxic capacity of FcγRIIib on neutrophils.\textsuperscript{12,26} It is conceivable that binding to the non-cytotoxic FcγRIIib decreases the cytotoxic capacity of the antibodies, by preventing them from binding to a cytotoxic FcγR.

No difference in membrane mobility of CD20 and HLA class II antigens
Finally, we analyzed whether a difference in lysis of HLA class II or CD20-sensitized Raji cells might be caused by a difference in mobility of the antigens in the cell membrane. If CD20 would indeed be less mobile than HLA class II, this could lead to a less efficient stimulation of the PMN, because clustering of FcγR on PMN is essential to trigger biologic responses.\textsuperscript{26} To test this hypothesis, we incubated Raji cells with chimeric CD20 mAbs or HLA class II mAb L243 with or without crosslinking antibodies (GAH and GAM respectively) for 4 hours. We evaluated the effect of crosslinking on the pattern of the membrane antigens by confocal microscopy. As is shown in fig. 5, upon crosslinking no difference was observed in clustering of HLA class II and CD20.
Fig. 5 No difference in clustering of CD20 and HLA class II upon crosslinking
Raji cells were incubated with chimeric CD20 mAbs or HLA class II mAb L243 (5 µg/mL) with (or without, not shown) crosslinking antibodies (50 µg/mL) for 30 min (A+B) or 4 hours (C+D) and cytospin preparations were made. Slides were stained with Texas Red phalloidin to detect F-actin (depicted grey) and FITC-labeled goat anti-human mAbs (GAH-FITC) or FITC-labeled goat anti-mouse mAbs (GAM-FITC) to detect chimeric CD20 mAbs and mAb L243 respectively (both depicted white).
Discussion

In the present study, we performed a detailed analysis on CD20-mediated cellular cytotoxicity of malignant B-cell lines and compared it with HLA class II-dependent ADCC. We found that, whereas non-primed neutrophils were not able to lyse antibody coated B-cells, G-CSF-primed PMN were capable of functioning as effector cells in CD20-dependent ADCC. However, HLA class II mAbs proved to be far more effective in inducing B-cell lysis.

Elsasser et al.\textsuperscript{12} consistently observed a lack of lysis of CD20-coated target cells by G-CSF-primed PMN. However, they used murine CD20 mAbs, in stead of the chimeric CD20 mAbs used in our experiments. Similarly, we did not observe any ADCC with murine (IgG2a) CD20 mAbs (see fig. 1). Because chimeric mAbs have previously been shown to be more effective in inducing ADCC than murine mAbs\textsuperscript{5,10}, this may explain the difference between our study and previous reported data on CD20-dependent ADCC.\textsuperscript{12}

Because antigen density of CD20 and HLA class II on Raji or Ramos cells was only slightly different (as demonstrated by FACS analysis, data not shown), in agreement with other reports\textsuperscript{12} we consider this not to be a likely explanation for the observed difference in ADCC-capacity.

Simultaneous sensitization of B-cells with (apoptosis inducing) F(ab')\textsubscript{2} fragments of HLA class II mAb L243 and CD20 mAbs did not increase the CD20-dependent ADCC. Hence, we could exclude HLA class II-induced apoptosis as an explanation for the high HLA class II-mediated ADCC. At the same time, these experiments decrease the probability of other (previously suggested) mechanisms by which ligation of HLA class II might render B-cells more sensitive to PMN-mediated killing (such as the upregulation of adhesion molecules on target cells or the induction of the production of TNF-\alpha, which is known to increase the cytotoxicity of neutrophils).\textsuperscript{12}

Conversely, since CD20 mAbs did not inhibit HLA class II-induced ADCC, we also excluded that ligation of CD20 by itself decreased B-cell susceptibility to PMN-mediated ADCC.

Recently, it was observed in mice that the function of activating Fc\gammaR in ADCC could be modulated by the inhibiting Fc\gamma receptor Fc\gammaRllb.\textsuperscript{27} Although mRNA encoding Fc\gammaRllb has been found in PMN\textsuperscript{28,29}, it is not known whether human neutrophils express Fc\gammaRllb. However, our observation that the level of HLA class II mediated ADCC was not affected by sensitization of B cells with both CD20 mAbs and HLA class II mAbs, may exclude downregulation of neutrophil response via interaction of the inhibiting Fc\gammaRllb with the Fc-tail of the chimeric CD20 mAb.

Relative immobility of an antigen in the cell membrane of the target cell could lead to less efficient Fc\gammaR-crosslinking mediated by the bound antibody and, consequently, to a less close contact between target cell and neutrophil. Therefore, we investigated the capacity of the CD20 and HLA class II antigens to cluster in the membrane. Since crosslinking of CD20 and HLA class II mAb L243 (with GAH and GAM antibodies respectively) induced an...
equal pattern of clustering and capping of the HLA class II and CD20 antigens, a difference in mobility was not likely to be the explanation for the difference in ADCC-activity of the antibodies.

In summary, we could exclude that the observed differences between HLA class II- and CD20-mediated PMN-ADCC were due to: 1) the use of chimeric (hlG1) mAbs versus mlgG2a mAbs; 2) HLA class II-induced apoptosis as an 'ADCC sensitizing' mechanism; 3) CD20-induced inhibition of ADCC and 4) inferior membrane mobility of CD20.

We did however observe a difference in the role of the different FcγR's in HLA class II- and CD20-dependent ADCC. CD20-induced PMN-ADCC was mediated mainly via FcγRI, although FcγRI and FcγRII were both required for optimal cytotoxicity (~20% lysis). In contrast in HLA class II-dependent ADCC, FcγRI and II were independently capable of inducing the maximum amount of lysis (~70%).

These observations suggest, that triggering of the effector functions of FcγRI and II by CD20 mAbs may be not optimal, at least when compared to HLA class II mAb L243. The underlying mechanism remains to be elucidated.

CD20 and HLA class II-induced MNC-ADCC were similar and, in line with previous studies\textsuperscript{10,12}, NK cells were found to be the main effector cells within the mononuclear cell fraction. Thus, the difference between CD20 and HLA class II mAbs with respect to triggering of the biologic effector functions of FcγRI and II apparently does not exist for FcγRIIa.

Our data demonstrate that chimeric CD20 mAbs capable of inducing ADCC with G-CSF primed neutrophils as effector cells. Still, NK cells were more potent inducers of CD20-dependent cytotoxicity. Recently, the importance of FcγR-mediated mechanisms for \textit{in vivo} cytotoxicity of monoclonal antibodies, including rituximab, was clearly demonstrated.\textsuperscript{30} Thus, stimulating the effector function of NK cells might be a promising way to enhance the efficacy of rituximab therapy. Indeed, it has previously been demonstrated in a nude mice model, that the combination of CD20 mAbs and IL-2 resulted in a larger decrease of tumor growth rates than CD20 mAbs alone.\textsuperscript{31} Furthermore, a recent clinical trial in which chimeric CD20 mAbs (rituximab) were combined with IFN-α showed that this combination might lead to a prolonged duration of response.\textsuperscript{32} However, cytokines used for \textit{in vivo} NK cell stimulation such as IL-2 and IFN-α often induce serious toxicity\textsuperscript{33-37}, as was observed in the described clinical trial as well.\textsuperscript{32} In contrast, G-CSF is a relatively mild therapy, able to induce large numbers of FcγRII-positive neutrophils. Therefore, the combination of rituximab with G-CSF is likely to be better tolerated.

More \textit{in vivo} studies should be performed to investigate the relative contribution of the different mechanisms of action of rituximab, in order to develop the best strategies to increase its clinical efficacy.
Reference List


