Rituximab-mediated antibody-dependent cellular cytotoxicity against neoplastic B cells is stimulated strongly by interleukin-2

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Background and Objectives. We analyzed the sensitivity of freshly isolated neoplastic B cells to rituximab-mediated antibody-dependent cellular cytotoxicity (ADCC), using different effector cells.

Design and Methods. ADCC was performed by 51Cr release assays *in vitro*, using peripheral blood mononuclear cells, IL-2-activated or expanded NK cells, neutrophils or macrophages as effector cells. B lymphoma lines and freshly isolated leukemic samples were used as targets.

Results. NK cells, but PMN or macrophages mediated rituximab dependent cellular cytotoxicity against two B lymphoma lines. Purified NK cells (95% $CD56^+/CD16^+$) reached 70% lysis at the highest E:T ratio. By contrast, all freshly isolated B leukemia or lymphoma cases, including 5 chronic lymphocytic leukemia, 1 B-prolymphocytic leukemia, 1 mantle cell lymphoma, 2 marginal zone lymhomas and 2 follicular lymphomas were poorly lysed by ADCC in the same conditions and regardless of CD20 expression levels, reaching a mean of 4% and 27% maximal lysis with PBMC or purified NK cells, respectively. Interestingly, short term IL-2 cultured PBMC, containing 10 % activated NK cells, as well as long-term expanded NK cells, containing 80-95% activated NK cells, became strong ADCC effector cells with rituximab and lysed all leukemic samples to a mean of 57% and 67% at the highest E:T ratio, respectively.

Interpretation and Conclusions. Primary leukemic cells are more resistant than cell lines to rituximab- and NK cell-mediated ADCC but short-term exposure to IL-2 or long-term expansion of NK cells *in vitro* may provide effective tools to improve the therapeutic activity of rituximab.

Key words: non-Hodgkin's lymphoma, natural killer, rituximab, antibody-dependent cellular cytotoxicity, IL-2.

Haematologica 2003; 88:1002-1012
http://www.haematologica.org/2003_09/1002.htm

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Over the last years several monoclonal antibodies have been approved for the treatment of patients with leukemia or lymphoma.[1] One of the most interesting of these, the anti-CD20 chimeric IgG1 rituximab, has shown clear-cut activity in B-cell neoplasia, achieving significant increases in clinical response rates in both low and high grade B non-Hodgkin's lymphomas (B-NHL) compared to treatment with chemotherapy alone.[2-5] Furthermore rituximab produces a 20% to 36% response rate in B-chronic lymphocytic leukemia cells (B-CLL) and shows a very favorable activity in combination with fludarabine.[6-8] However, a full understanding of its *in vivo* mechanism of action as well as optimization of its interaction with human effector cells and proteins are essential in order to improve the efficacy of treatment with rituximab. Recent evidence obtained both *in vitro* and *in vivo* in an animal model demonstrates that complement activation is required for the therapeutic activity of rituximab (*Di Gaetano et al., submitted*). On the other hand, another study showed that activating Fc receptors (FcγRI or FcγRIII or both) are also required, suggesting an alternative or additive role for antibody-dependent cellular cytotoxicity (ADCC).[9] Finally, the genetic analysis of Fcγ receptor polymorphism in populations of treated patients adds further evidence concerning the importance of ADCC in the response to rituximab *in vivo*.[10]

Several cell populations express activating FcγR and have been shown to be effector cells for ADCC: natural killer (NK) cells, polymorphonuclear cells (PMN) and macrophages.[11-14] Furthermore different cytokines have been shown to activate these effector cells.[15-18] In particular interleukin-2 (IL-2) has been shown to activate natural killer cell activity towards susceptible targets.[19,20] NK cell cytotoxic activity against natural targets is regulated by a fine balance between activating and inhibitory signals through NK cell receptors, in particular the killing inhibitory receptors (KIR) that bind to MHC class I molecules.[21] The ADCC activity of NK cells, on the other hand, is triggered by binding of immunoglobulin molecules to FcγRIII, but may also be regulated by other signals.[22,23] We have determined the ADCC activity of rituximab *in vitro* using different effector cell populations and two representative B-cell lines, as well as a panel of freshly isolated neoplastic B cells from different leukemia/lymphoma subtypes as targets.

Design and Methods

Cell purification and culture conditions

The B-cell lines BJAB (Burkitt's lymphoma) and MEC2 (B-CLL), and the erythromyeloid cell line K562 were cultured in RPMI1640 medium (Seromed, Berlin, Germany) supplemented with 10% fetal calf serum (FCS) (Sterile System; Hyclone Laboratories, Logan, UK) and 2 mM glutamine (Life Technologies Paisley, Scotland), 100U/mL penicillin and 100 μg/mL streptomycin (hereafter called RPMI complete medium). Peripheral blood mononuclear cells (PBMC) were purified from normal volunteers by standard Ficoll-Hypaque (Seromed) gradient centrifugation. The cells were either used immediately or cultured for a short period (48 hours) in RPMI complete medium in the presence of human recombinant interleukin 2 (hrIL-2 1,000 U/L; Serono, Rome, Italy).

PMN cells were purified from peripheral blood or buffy coats as described previously.[24] Briefly, cells were separated by Ficoll gradient centrifugation followed by purification on a 62% Percoll gradient (Amersham Biosciences, Cologno Monzese, Italy). Cells were washed and used immediately.

Highly purified $CD56^+/CD3^-$ NK cells were obtained by immunomagnetic isolation. Briefly, adherent cells were removed from PBMC by incubation in complete medium in plastic flasks for 1-2 hours at 37°C, and T cells were depleted by staining at 4°C with monoclonal antibodies against CD3 (clone OKT3) (American Type Culture Collection) and CD5 (clone OKT1) (American Type Culture Collection). After 30 minutes the cells were washed twice in PBS and incubated for 15 min at 4°C with 20 μL goat-anti-mouse microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany)/10^7 cells. The $CD3^-$ fraction was purified using an automatic magnetic-activated cell sorting system (AutoMACS system, Miltenyi Biotec, Bergisch Gladbach, Germany) and further separated into $CD56^+$ and $CD56^-$ cell fractions using anti-CD56 monoclonal antibody-coated magnetic beads (Miltenyi Biotec). The purity of the NK enriched populations was 95-99% $CD56^+/CD3^-$ NK cells as assessed by flow cytometry. Macrophages were obtained by *in vitro* differentiation of peripheral blood monocytes. Briefly, monocytes were purified by centrifugation on a 46% Percoll gradient and were cultured for 7 days in RPMI1640 medium supplemented with 20% FCS and 100 ng/mL M-CSF (Peprotech, Rocky Hill, NJ, USA).

Heparinized peripheral blood was obtained after informed consent from patients with B-chronic lymphocytic leukemia (B-CLL), prolymphocytic leukemia (PLL), mantle cell lymphoma (MCL), marginal zone lymphoma (MZL) and follicular lymphoma (FL) with significant circulating disease (at least 50% neoplastic cells in the mononuclear cell fraction). All patients were diagnosed by routine immunophenotypic, morphologic and clinical criteria. In all cases double staining with CD19 and slgκ or slgλ 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 gradient centrifugation and aliquots of cells were frozen in 10% dimethylsulfoxide according to standard procedures.

Expansion of NK cells in vitro (long-term expanded and activated NK cells)

PBMC were depleted of adherent cells as described above and co-cultured at 4×10^5/mL cells in the presence of irradiated (3000 rad) RPMI 8866 cells (an EBV^+ lymphoblastoid B-cell line) (1×10^5/mL) for 10-12 days at 37°C in a humidified 5% CO_2 atmosphere as previously described.[25] rhIL-2 (1000 U/mL) was added after 7 days. The expanded population of cells consisted of 60-90% $CD56^+/CD16^+/CD3^-$ NK cells and the number of cells increased by a mean of 10-fold during this period. In cases in which purity was below 80% following *in vitro* expansion, the cells were further purified by immunomagnetic positive selection using anti-CD56 magnetic microbeads (Miltentyi Biotec) and the AutoMACS. The resulting purified cell population was more than 97% $CD56^+/CD16^+/CD3^-$ as assessed by cytofluorimetric analysis.

Antibody-dependent cellular cytotoxicity (ADCC)

Target cells ($2\text{-}3\times10^6$) were labeled by incubation for 2 hours at 37° C in 300 μL complete medium containing 60 μCi sodium chromate^{51} (Amersham Biosciences). Cells were then washed and incubated in the presence or absence of the chimeric anti-CD20 antibody, rituximab (a kind gift from Roche Italia, Monza, Italy) at 10 μg/mL for 20 min at room temperature; the K562 cell target was used without antibody treatment. Cells were then washed and plated at 10^4/well in round-bottomed 96-well plates. Increasing amounts of effector cells were added to triplicate wells to reach effector:target (E:T) ratios ranging from 2:1 to 100:1, in a final volume of 200 μL. Control wells contained only target cells (to measure spontaneous release) or target cells with 1% SDS (total cpm). In some experiments recombinant human IFN-γ (500 U/mL) (Roussel-UCLAF, Paris, France) or GM-CSF (100 ng/mL) (Schering-Plough, Milan, Italy) was added to the assays. The plates were incubated for 5-6 hours at 37°C in a CO_2 incubator. Then, 100 μL of supernatant were collected from each well and counted in a gamma-counter. Percentage lysis was calculated after substracting the cpm due to spontaneous release (in the absence of effector cells) from all samples and using the following formula: cpm test sample ×100/total cpm.

Table 1. CD20 expression of neoplastic B cells.

Patient/ cell line	*% CD20*	*MFI CD20*
Cell line		
BJAB	99	410
MEC2	99	530
Patient		
CLL1	95	750
CLL2	90	87
CLL3	71	68
CLL4	74	250
CLL5	94	444
PLL1	91	1183
MCL1	83	860
MZL1	96	1408
MZL2	92	660
FL1	51	932
FL2	82	200

MFI: mean fluorescence intensity.

Immunofluorescence analysis

Cells were analyzed by standard direct immunofluorescence, using a FACScan (BD Biosciences, Heidelberg, Germany) and phycoerythrin- or fluorescein-conjugated antibodies against CD3, CD56, CD16 and CD20 (BD Biosciences). The fluorescence analyses of the two cell lines were performed in parallel. In the case of freshly isolated leukemic cells, samples could not always be analyzed at the same time. Therefore, as a routine, we have performed these analyses setting the peak of the negative control between the first and tenth channel of fluorescence to obtain fluorescence profiles, that although not strictly quantitatively equal, were at least roughly comparable with each other.

Results

NK cells are responsible for rituximab-mediated ADCC when PBMC are used as effector cells

In order to identify the more active effector cell population for anti-CD20 rituximab-mediated ADCC activity against B-cell leukemias/lymphomas, we selected as targets two human CD20+ B-cell lines; the Burkitt's lymphoma BJAB cell line and the B-CLL derived MEC2 cell line. Both express CD20 to a similar level (MFI 410 and 530, respectively)(Table 1 and Figure 1, panel A).[26,27] Several different cell preparations, including PBMC, PMN and monocyte-derived macrophages, were then tested as effector cells in ADCC assays at different E:T ratios. In addition, in some cases interferon-γ (IFN-γ) (500 U/mL) or GM-CSF (100 ng/mL) were added to the assays. As indicated in Figure 1, panel B, PBMC showed significant ADCC activity against both B-cell lines in the presence of rituximab (up to 38% and 46% lysis, respectively), in sharp contrast with the observed cytotoxicity values in the absence of antibody (10% and 0% lysis respectively). By contrast, PMN, purified to 95% by density gradient centrifugation, did not mediate significant ADCC against either line, even at the highest E:T ratios. Furthermore, performing the ADCC assays in the presence of activating doses of IFN-γ (500U/mL) or GM-CSF (100 ng/mL) did not increase target cell killing by PMN. Similar negative results were obtained with *in vitro* differenti-

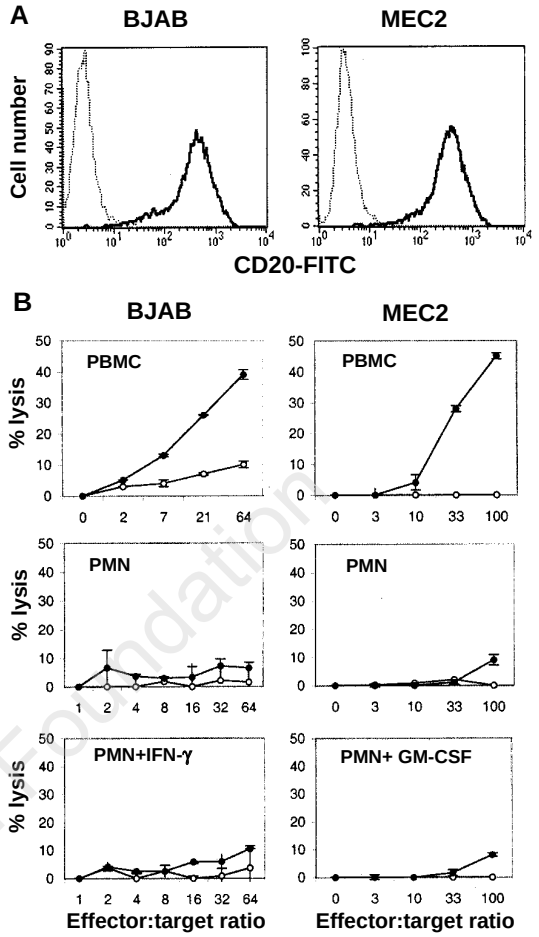

Figure 1. Rituximab-mediated ADCC of B lymphoma cell lines using different effector cell populations. Panel A: Immunophenotype of the BJAB and MEC2 lymphoma cell lines using FITC-labeled anti-CD20 (thick lines) or IgG1 control antibody (thin lines). Panel B: ADCC assays of the BJAB and MEC2 cell lines in the presence (closed circles) or absence (open circles) of rituximab, using PBMC or PMN as effector cells at the indicated effector:target ratios. Some assays were performed in the presence or absence of IFN-γ (500 U/mL) or GM-CSF (100 ng/mL), as indicated in each panel. The results are representative of at least two independent experiments for each cell line and using 4 different donors.

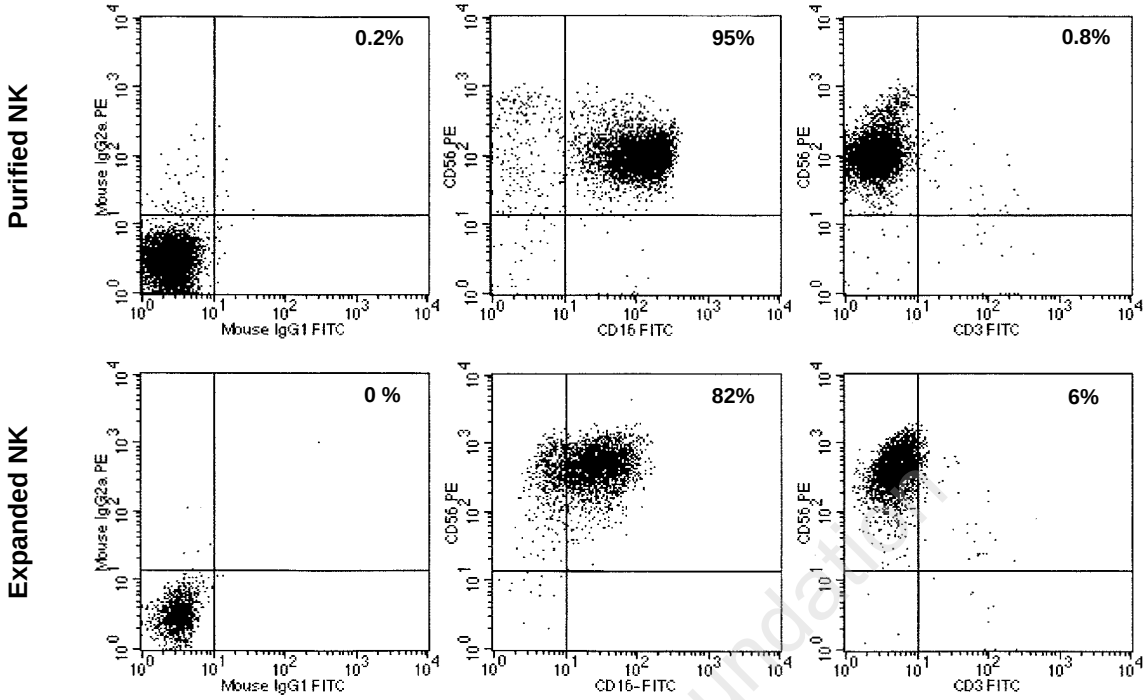

Figure 2. Phenotype of peripheral blood or *in vitro*-expanded purified NK cells. NK cells were purified from buffy coats by consecutive negative and positive selection using immunomagnetic beads (upper panels). NK cells were expanded by co-culture of PBMC with the 8866 lymphoblastoid cell line in the presence of rhIL-2 (lower panels). Double immunofluorescence analyses of the purified populations using CD16-FITC/CD56-PE, CD3-FITC/CD56-PE or control antibodies are shown. The data are representative of at least 4 different experiments with at least 4 different donors.

ated macrophages (*data not shown*). That the monocytes had differentiated to macrophages was verified by their expression of CD16, the FcγRIII molecule, which was found to be present on 91% of the cells (*data not shown*).

These data suggest that NK cells are the main effector population in executing rituximab-mediated ADCC against B-cell targets, since $CD56^+$/$CD16^+$ NK cells accounted for a mean of 10% (standard deviation 2.7) of the cells in our PBMC preparations (*data not shown*). In order to confirm this hypothesis, $CD56^+$/$CD16^+$ cells were purified from buffy coats to 95-98% purity by successive positive and negative immunoselection (Figure 2, upper panel). These cells did not express CD3 (Figure 2) and efficiently lysed the classical NK target cell line K562 (Figure 3A) showing that they were true NK cells. As expected, purified NK cells were extremely active in ADCC assays against the MEC2 cell line, showing over 70% lysis in the presence of rituximab at the highest E:T ratio compared to 17% when the antibody was absent (Figure 3B). We conclude that NK cells are the main active primary effector cell population against B lymphoma/leukemia cell lines in rituximab-mediated ADCC assays, at least under the assay conditions tested here.

Freshly isolated neoplastic B cells are poor targets of rituximab-mediated ADCC by NK cells

We then tested 11 freshly isolated cases of B-cell lymphoma/leukemia in ADCC assays using PBMC. The level of CD20 expression of all cases, given in terms of MFI, is shown in Table 1. The B-CLL samples chosen for the analysis contained an unusual proportion of cases (2 out of 5) with high CD20 expression levels (MFI of 750 and 444, respectively, Table 1). These were deliberately selected out of a much larger panel of B-CLL samples in order to analyze ADCC activity of rituximab on B-CLL samples showing both high and low expression levels of CD20 and exclude the possibility that negative results could be the consequence of low CD20. The other leukemic samples were unselected.

Figure 4 shows the results obtained in 4 representative cases which expressed high (PLL1), intermediate (CLL1) or low levels of CD20 (CLL2 and

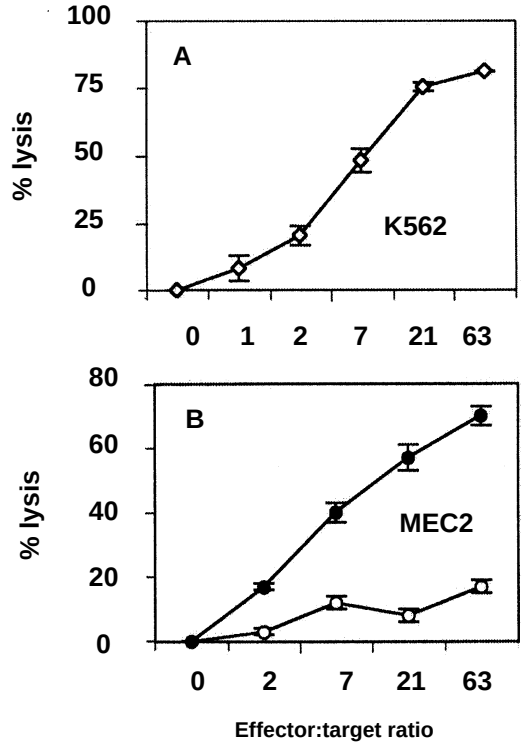

Figure 3. NK and ADCC activity of purified peripheral blood NK cells on target cell lines. NK cells purified from buffy coats were used as effector cells at the indicated effector:target ratios on the K562 cell line (open diamonds) or on the MEC2 B-cell line in the presence (closed circles) or absence of rituximab (open circles). The results are representative of at least 2 experiments for each target cell line, using at least 4 different donors.

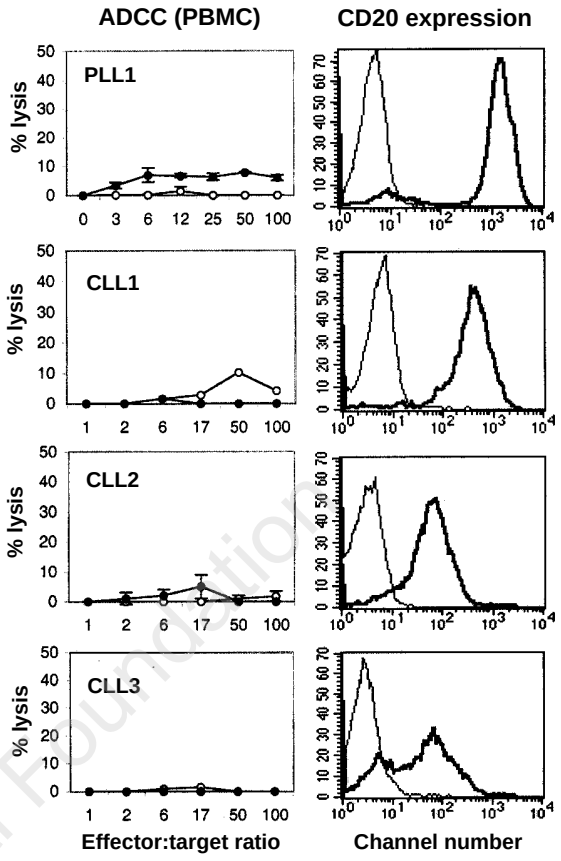

Figure 4. Rituximab-mediated ADCC activity of PBMC on freshly isolated B-cell leukemias. ADCC assays were perfomed on the indicated freshly isolated leukemic samples using PBMC as effector cells in the presence (closed circles) or absence of rituximab (open circles). The right panels show the results of immunophenotypic analysis of the B leukemia samples using FITC-labeled anti-CD20 antibody. The results were obtained using at least 4 different PBMC donors.

CLL3)(Figure 4 and Table 1). In all cases analyzed, only slight cytotoxicity, reaching a maximum of 8% at the highest E:T ratio, could be detected (Figure 4 and see below). Thus, using the same experimental conditions and effector cell populations, i.e. PBMC, freshly isolated B leukemia/lymphoma cells are much more resistant to rituximab-mediated ADCC than are B-cell lines, even when equivalent levels of CD20 are expressed in both cases (Table 1).

In order to determine whether resistance could be overcome by using pure populations of NK cells, purified NK cells from normal donors were tested on two selected leukemic samples (PLL1 and CLL2). As shown in Figure 5, purified NK cells were able to lyse the target B cells to a maximum of 25 and 30%, respectively, at the highest E:T ratio. Thus lysis was measurable using purified NK cells on freshly isolated leukemic B cells, although it was considerably weaker (by 60-70%) than that observed using the same population of effector cells and the MEC2 cell line as the target (Figure 3B). Similar data were obtained with purified NK cells from 2 different donors (*data not shown*). These data suggest that peripheral blood-derived NK cells are indeed able to mediate ADCC against $CD20^+$ leukemic cells, but with an efficiency well below that obtained using cell lines.

Short-term IL-2-activated NK cells mediate strong ADCC against freshly isolated leukemic samples

NK cells have long been known to be strongly activated by IL-2, giving rise to the so-called lymphokine activated killer (LAK) cells able to kill NK cell targets very efficiently.[21] However the effect of IL-2 on the ADCC activity of NK cells has been lit-

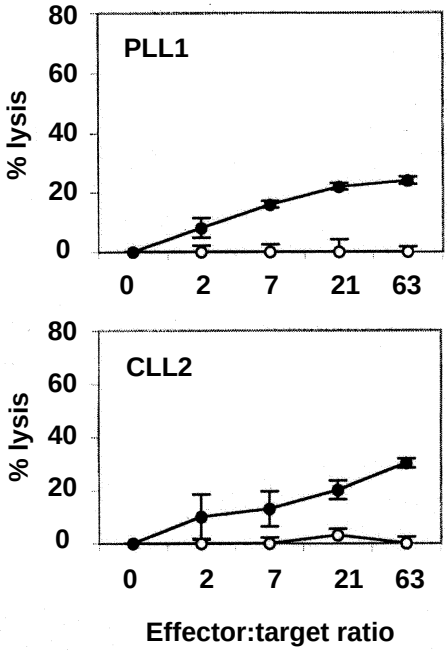

Figure 5. Rituximab-mediated ADCC activity of purified peripheral blood NK cells on freshly isolated B leukemias. NK cells purified from buffy coats were used as effector cells at the indicated effector:target ratios on two representative freshly isolated B leukemia cases in the presence (closed circles) or absence of rituximab (open circles). The results are representative of 2 experiments for each target with a total of 3 different donors.

tle investigated. Total PBMC were cultured for 2 days in the presence of rhIL-2 and tested against the classical NK target K562 in the absence of antibody, as well as against the two B-cell lines in the presence or absence of rituximab. As shown in Figure 6, two days' exposure to IL-2 were sufficient to induce a dramatic increase in cytotoxicity against the K562 target and the two B-cell lines to such strength (80% lysis at low E:T ratios) that the effect of the addition of the anti-CD20 antibody to the B cell lines was not measurable (Figure 6). These results, therefore, show that IL-2 dramatically increases NK activity by PBMC against cell lines (Figure 6). Phenotypic analysis performed on several PBMC populations demonstrated that short-term exposure to IL-2 did not significantly change the percentage of $CD16^+/CD56^+$ NK cells in the culture, nor did it lead to a significant increase in $CD56^+/CD3^+$ cells (*data not shown*) suggesting that the increased cytotoxicity was due to NK cell activation rather than expansion.[21]

The IL-2-activated PBMC populations were then tested against 9 freshly isolated B-cell leukemia/lymphoma samples. The cases included 3 B-CLL, 1 PLL, 1 MCL, 2 MZL and 2 FL. In all cases the rituximab-mediated cytotoxicity detected before IL-2 exposure was negligible, reaching a mean of 4% lysis above background at the highest E:T ratios (Figures 7A and B, upper panels). Exposure to IL-2 for 48 hours clearly increased NK activity in the absence of antibody (detectable in 7/9 cases) as well as rituximab-mediated ADCC. Indeed, total lysis in the presence of antibody reached 36-67% at the highest E:T ratio (mean 57%). This lysis was 15-48% higher (mean 29%) than that observed in the absence of antibody (Figures 7A and B). Thus rituximab further increases the cytotoxic activity of IL-2-activated NK cells on freshly isolated B leukemia targets. It is worth noting that in two cases in which natural cytotoxicity (in the absence of rituximab) was absent (PLL1 , MZL2), the addition of antibody mediated a strong ADCC reaction (up to 48 and 36%, respectively)(Figures 7A and B). Nonetheless, the cytotoxic activity of activated NK cells against leukemic samples, although strong, remained below that observed with the same effector populations against the cell lines as targets, when lysis reached 80-90% (Figure 6).

These data strongly suggest that, whereas PBMC are poor effector cells in killing primary leukemic B cells in presence of rituximab, the same cells become strong effectors after short-term exposure to IL-2.

Cytotoxic activity of long-term in vitro expanded and activated NK cells

To substantiate the positive role of activated NK cells in expleting rituximab-mediated cytotoxicity, we expanded and activated NK cells *in vitro* using feeder cells, as described previously.[25] The phenotype of one such expanded population is shown in Figure 2 (lower panel), demonstrating that >80% of the cells were $CD56^+/CD16^+$ and did not express CD3. These cells were CD56 bright and also expressed CD16 (Figure 2).[20] As expected, the expanded NK population showed very strong activity against the K562 and MEC2 cell lines (Figure 8). In both cases 60% specific lysis was detected at a 1:1 E:T ratio and 100% lysis of MEC2 cells could be observed in the presence of rituximab at a 32:1 E:T ratio. When tested against freshly isolated leukemic cells, strong antibody-dependent cytotoxicity was observed, reaching 60-75% at highest ratios, and lysis increased by 24-44% in the presence of the antibody. Lysis obtained with expanded NK cells was comparable to that obtained with PBMC after short-term exposure to IL-2 (Figures 7 and 8). These data show that NK cells can be rapidly expanded *in vitro* and are then capable of strong ADCC activity against both leukemic B-cell lines and freshly isolated samples. As with other effector cell populations, the susceptibility of fresh samples to lysis remains inferior to that detected with the cell lines.

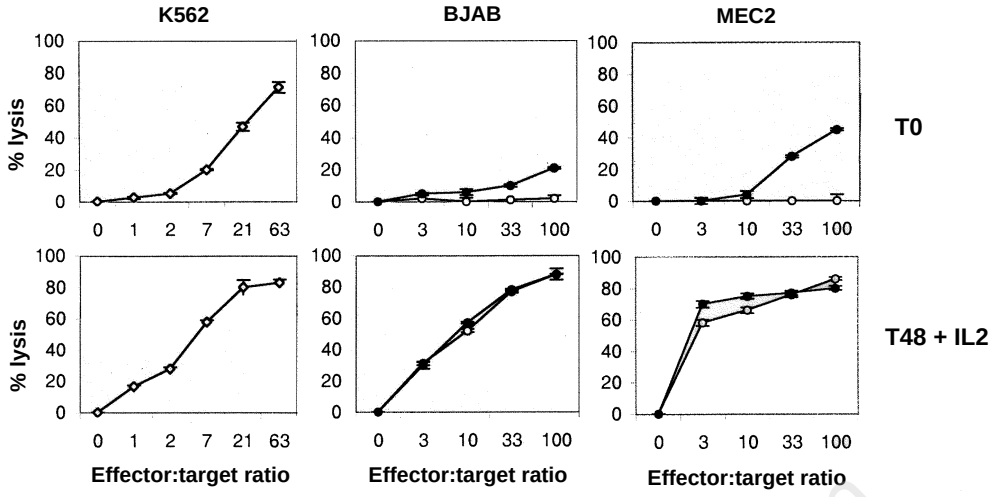

Figure 6. IL-2 dramatically increases the NK and ADCC activity of PBMC against cell lines. PBMC from the same donors were either used immediately as effector cells in NK and ADCC assays or after 2 days' culture in the presence of rhIL-2. The target cells used were a ^{51}Cr-labeled K562 cell line as the NK cell target or the BJAB and MEC2 B cell lines in the presence (closed circles) or absence (open circles) of rituximab. The results are representative of a total of 4 experiments with 4 different donors.

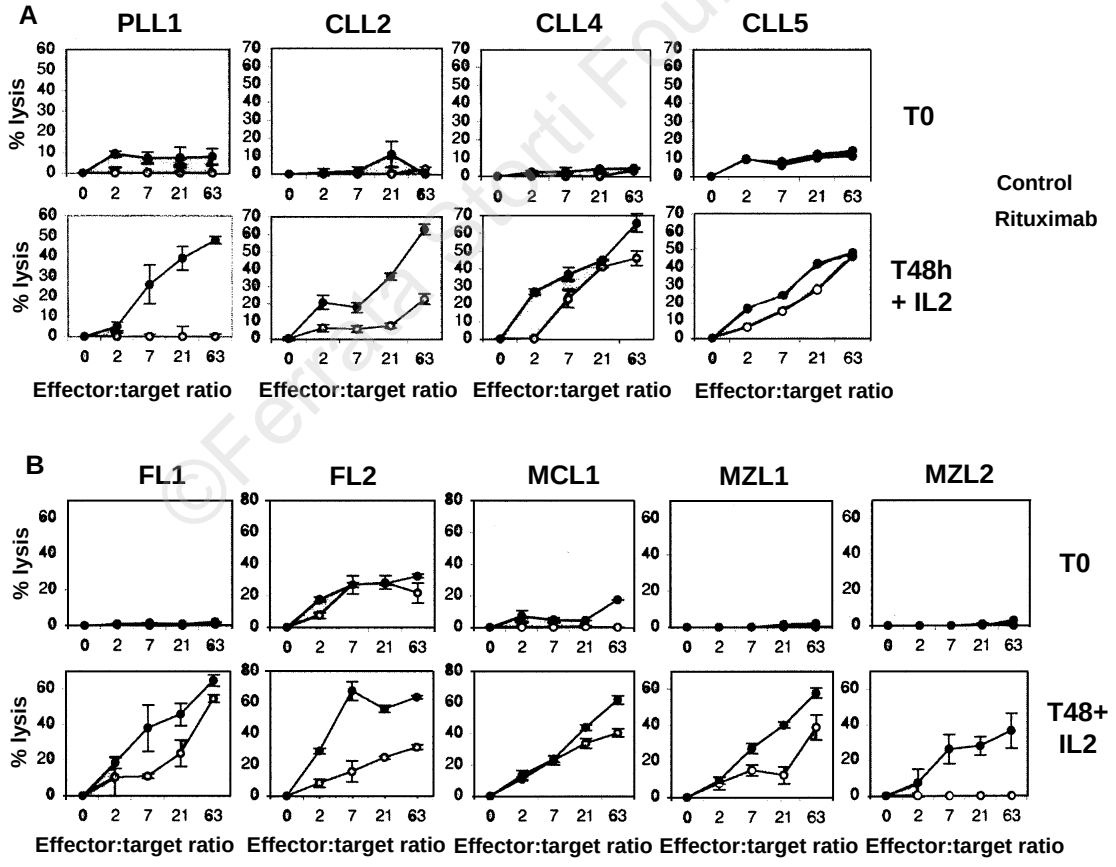

Figure 7. IL-2 dramatically increases the NK and ADCC activity of PBMC against freshly isolated leukemias. PBMC from the same donors were either used immediately as effector cells in ADCC assays or after 2 days' culture in the presence of rhIL-2. The target cells used were the indicated ^{51}Cr-labeled B-PLL or B-CLL samples (panel A) or FL, MCL or MZL samples (panel B) in the presence (closed circles) or absence (open circles) of rituximab. The experiments shown are representative of a total of at least 15 experiments with at least 12 different donors.

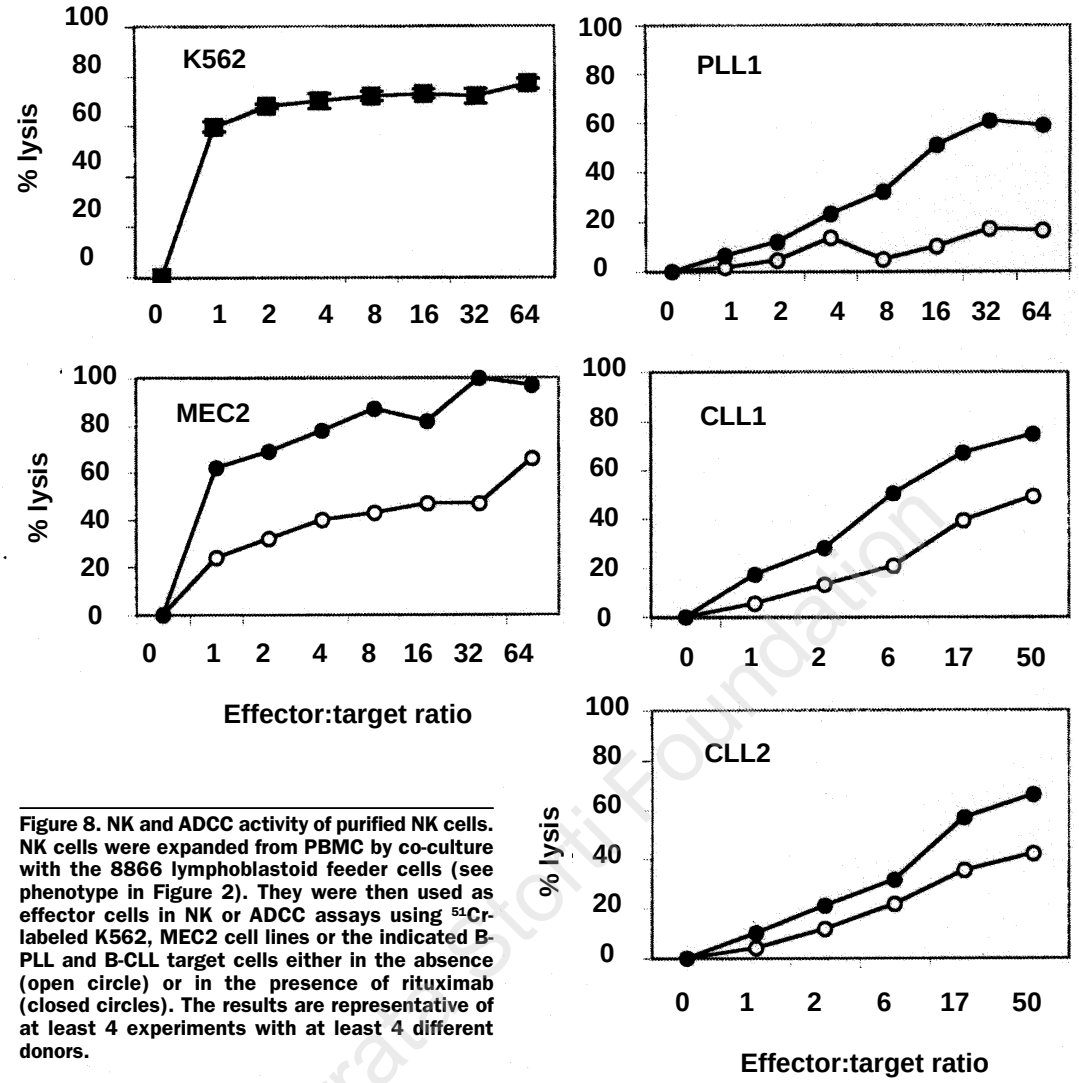

Figure 8. NK and ADCC activity of purified NK cells. NK cells were expanded from PBMC by co-culture with the 8866 lymphoblastoid feeder cells (see phenotype in Figure 2). They were then used as effector cells in NK or ADCC assays using ^{51}Cr-labeled K562, MEC2 cell lines or the indicated B-PLL and B-CLL target cells either in the absence (open circle) or in the presence of rituximab (closed circles). The results are representative of at least 4 experiments with at least 4 different donors.

Discussion

The *in vivo* mechanism of action of the chimeric anti-CD20 IgG1 monoclonal antibody, rituximab, is still a matter of debate, in spite of its proven therapeutic efficacy in the treatment of B-cell tumors.[26-29] Rituximab has been shown to activate complement efficiently both *in vitro* and *in vivo* and recent data suggest that complement activation is required for the therapeutic activity of rituximab.[26,30] Additional mechanisms, in particular ADCC,[31] are also likely to play a role. Indeed rituximab loses full therapeutic activity in an *in vivo* model in nude mice following FcγR chain knock out, suggesting a role for either FcγRI or FcγRIII or both.[9] Furthermore, genetic analysis of FcγRIIIA polymorphisms in B-NHL patients treated with rituximab alone suggests that this receptor is involved in the clinical response to the antibody.[10] This last study is of particular intertest in view of the fact that the more favorable allotype has been shown to be associated with better binding to IgG1.[32] We and others have previously shown that rituximab can mediate ADCC activity against B-cell lines.[27,33] In the present study, we report the activity of different effector populations on both freshly isolated leukemic cases and cell lines.

Our data show that freshly isolated PBMC are the best source of effector cells to mediate rituximab ADCC *in vitro* against cell lines, whereas PMN and macrophages are inactive, at least under the standard ADCC conditions used here. That the 10% NK cells present in PBMC were responsible for ADCC was shown by purification of these cells. Purified peripheral blood NK cells were indeed very active in mediating ADCC against the cell lines.

PMN have been shown to be able to mediate ADCC with some but not all antibodies,[13,23,34,35] but lysis is most effective when PMN are isolated from G-CSF treated individuals or when bispecific monoclonal antibodies, directed against either the FcγRI or FcαRI molecules on the effector cells, are used.[14,22,36] Thus our results with PMN are consistent with previous reports using rituximab[31,35] and show that even *in vitro* stimulation with GM-CSF or IFN-γ is not sufficient to induce ADCC activity by PMN with this antibody, in contrast to observations with some other monoclonal antibodies.[13,37] The reason for the lack of activity of PMN in the presence of rituximab is not clear; the capacity of different antibodies to activate PMN ADCC does not seem to depend upon the Fc portion but rather on the particular target antigen.[22,35]

The second conclusion we reached is that freshly isolated leukemic cells were less sensitive than B lymphoma lines to *in vitro* ADCC. Indeed, we could not detect significant killing of primary leukemic cells using PBMC as effectors, in conditions in which lysis of 30-40% of cell lines could be observed. This was true regardless of antigen density on the target, suggesting that it was not simply due to low CD20 expression. Even though measurable ADCC (reaching 25-30%) could be detected using purified NK cells, in agreement with a previous report,[31] this still required high effector:target ratios (63:1) and was well below that observed using cell lines (70-80% lysis). Thus, although primary leukemic B cells can be killed by NK cells, they are more resistant to lysis than are cell lines. The molecular basis for this resistance is still unclear. These results suggest that caution should be exercised when drawing conclusions from study of cell lines only. It is possible that ADCC by NK cells may be more effective *in vivo* than *in vitro*, for example following cytokine induction *in vivo*[38] or through synergism between complement activation and ADCC after binding of complement fragments to the appropriate receptors on effector cells.[39,40] On the other hand the relative number of NK cells in the blood of leukemic patients is very low. Indeed we have been unable to detect a significant number of NK cells in the lymph nodes of patients with follicular lymphoma (*Golay J., unpublished observations*), making these findings difficult to reconcile with an important role for ADCC in the mechanism of action of rituximab *in vivo*, at least on the basis of the *in vitro* activity of NK cells described here.

PBMC cells activated *in vitro* by short-term exposure to IL-2 become strong effectors in rituximab-mediated ADCC against primary leukemic samples. NK activity was also significantly increased in the absence of antibody. That the cells responsible for ADCC were NK cells was confirmed by expansion and purification of IL-2-activated NK cells showing that this population was indeed extremely active in the presence of rituximab. Some background NK activity in the absence of rituximab could be detected in 7/9 cases, as expected, presumably due to a mismatch between the KIR receptors on NK cells and the MHC molecules on the leukemic targets.[41] However, rituximab significantly increased the killing by IL-2-activated PBMC or purified NK cells in all primary leukemic samples examined. As expected from the short-term exposure to IL-2, increased ADCC was not due to an increase in the number of NK cells but rather to their functional activation.[19,21] This suggests that rituximab can overcome the negative signals by KIR receptors in IL-2-activated NK cells.[21,41] These data offer a biological rationale for the combined use of rituximab and IL-2 *in vivo*. Recent reports already suggest the good activity and safety of this combination in patients with relapse of their disease.[42]

More importantly, our data suggest that NK cell activation and/or expansion *in vitro* can be performed and that this, if carried out in clinical grade conditions, may offer an alternative therapeutic protocol to direct IL-2 infusion in conjunction with rituximab. The feasibility of expanding NK cells from a B-CLL patient, which could then kill the autologous leukemic cells highly efficiently *in vitro*, has already been shown by one of us (*R Broady, unpublished data*). Attention must, however, be given to the regulatory role of NK cells or other immune cells which may affect the overall activity of NK cells *in vivo*.[43] While an experimental model is currently been studied in our laboratory, clinical trials have already explored the feasibility of treating cancer patients with either NK or LAK cells: the results are promising.[44]

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Pre-publication Report & Outcomes of Peer Review

Contributions

JG, MM, RG and VF carried out the ADCC experiments; RB and GB purified and characterized the NK cells; JG, AR and MI contributed to the conception and design of the study and obtained funding; AR also provided leukemic samples; JG and MI analyzed the data and wrote the manuscript.

Primary responsibility for the paper: JG; primary responsibility for Table 1: JG; primary responsibility for Figure 1: JG, VF; primary responsibility for Figure 2: MM; primary responsibility for Figure 3: GB, RB; primary responsibility for Figures 4 and 5: JG; primary responsibility for Figures 6 and 7: MM, JG; primary responsibility for Figure 8: RB, MM.

Funding

This work was supported by the Italian Ministry for University and Research (projects FIRB no. RBAU01J2ER and no. RBAU01H8SX), the Associazione Italiana Ricerca sul Cancro (AIRC), Roche Italia (Monza, Italy), the Istituto Superiore di Sanità (ISS Rome, project 30D.41) and by the Associazione Paolo Belli-Lotta alla Leucemia, Bergamo, Italy. MM is the recipient of a fellowship from the Associazione Italiana contro la Leucemia (AIL).

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received July 15, 2003; accepted August 5, 2003.

In the following paragraphs, Professor Cazzola summarizes the peer-review process and its outcomes.

What is already known on this topic

Although rituximab is effective in the treatment of B-cell lymphomas, the *in vivo* mechanism of action of this chimeric anti-CD20 monoclonal antibody is still a matter of debate. In addition, since only a portion of patients respond to treatment, there is a need to improve the therapeutic activity of this novel drug.

What this study adds

The findings of this study suggest that exposure to interleukin-2 or long-term expansion of NK cells *in vitro* may provide effective tools to improve the therapeutic activity of rituximab.