We have investigated the capacity of polymorphonuclear phagocytes (PMN) to lyse malignant B-cell lines using antibodies and antibody derivates to a range of different B-cell antigens. PMN were found to mediate lysis of all tested B-cell lines in the presence of HLA class II antibodies L227, L243, F3.3, and CR2/43. Target cell lysis was significantly enhanced when PMN isolated during granulocyte colony-stimulating factor (G-CSF) treatment were compared with PMN from healthy donors. Only G-CSF primed PMN, expressing FcγRI (CD64), lysed B cells in the presence of monoclonal antibody (MoAb) 1D10 or Lym-1 to HLA class II related epitopes. Remarkably, PMN were consistently unable to kill malignant B cells with antibodies to the B-cell related antigens CD19, CD20, CD21, CD37, and CD38. This target antigen restriction was not observed with mononuclear effector cells, which mediated cytotoxicity with antibodies to HLA class II, but also with mouse/human chimeric constructs to CD19, CD37, and CD38. Blocking studies with FcγRI antibodies and reverse antibody-dependent cellular cytotoxicity (ADCC) experiments against FcγRI antibody expressing hybridoma targets confirmed the pivotal role of FcγRI in enhanced killing by G-CSF primed neutrophils. Bispecific antibodies (BsAb) with one specificity for FcγRI, and another for a tumor associated antigen, offer an interesting approach to improve effector cell recruitment for immunotherapy. In our studies, very effective lysis was observed with G-CSF primed PMN and an [HLA class II × FcγRI] BsAb. The therapeutic implications of these findings and the possible use of BsAb in combination with G-CSF are discussed.

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Toxic trigger molecules on effector cells. A phase I study with such a bispecific antibody (BsAb) to FcyRI and HER-2/neu on breast cancer cells has recently been finished, and other trials with the same antibody in combination with G-CSF, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon (IFN)-γ are in progress.

**MATERIALS AND METHODS**

**Cell lines.** The malignant B-cell lines REH (0-acute lymphoblastic leukemia [ALL]), RAJI (Burkitt’s lymphoma), BALL (B-ALL), SB and CESS (both Epstein-Barr virus [EBV] transformed B-cell lines), and the undifferentiated myeloid cell line KG-1 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). RM-1 (EBV transformed B-cell line) and BJAB (Burkitt’s lymphoma) were from G. Bonnard (Bethesda, MD) and W. Leibold (Department of Veterinary Medicine, Hannover, Germany), respectively. All cells were kept in RPMI 1640 (GIBCO, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, 100 U/mL streptomycin, and 4 mmol/L L-glutamine (GIBCO).

**Monoclonal antibodies (MoAb) and antibody constructs.** Hybridomas of HLA class II antibodies L243 (mlgG2a), L227 (mlgG1), and Lym-1 (mlgG1) were from ATCC, CR3/43 (mlgG1) was from Dako (Glostrup, Denmark), HLA class II antibodies F3.3 (mlgG1) and ID109 (mlgG2a) were produced in our laboratories (M.GI. and G.W., respectively). Murine antibodies for immunofluorescence and sensitization of malignant B cells were J4.119 (CD19, mlgG1; Becton Dickinson, San Jose, CA), CLB-CD19 (CD19, mlgG1 and its natural IgG2a switch variant, S. Vervoordeldonk, CLB, Amsterdam, The Netherlands), HD37* (CD19, mlgG1; Immunotech), WR17 (CD19, mlgG1; Becton Dickinson, San Jose, CA), and negative control antibody TIB92 (mIgG2a, ATCC). Target antibodies against CD37, CD38, and CD40 from their parental antibodies.

**Cytokines.** Patients were treated with rh-met-G-CSF (Neupogen, 3 to 5 μg/kg of body weight) from Hoffmann La-Roche (Basel, Switzerland), based on clinical indications.

**Blood donors.** Experiments reported here were approved by the Ethical Committee of the University of Erlangen-Nürnberg (Germany), in accordance with the Declaration of Helsinki. After informed consent, 10 to 20 mL of peripheral blood was drawn from healthy volunteers or patients receiving G-CSF therapy. As the kinetics of neutrophil recovery during G-CSF treatment vary, the following criteria were defined for analysis: (1) at least 3 days of G-CSF treatment and (2) absolute neutrophil count >2,500/μL. RFI expression for FcyRI (CD64), FcyRII (CD32), and FcyRIII (CD158) was determined by indirect immunofluorescence and characterized by high performance liquid chromatography (HPLC). Samples were sterilized by filtration and stored at -20°C. All BsAbs showed binding to effector and target cells as expected from their parental antibodies.

**Isolation of mononuclear and neutrophil effector cells.** Neutrophils were isolated by a method slightly modified from that previously described. Briefly, citrate anticoagulated blood was layered over a discontinuous percoll (Seromed, Berlin, Germany) gradient consisting of 70% and 62% for healthy donors, and of 68% and 59% of percoll for G-CSF recipients, respectively. After centrifugation, neutrophils were collected at the interphase between the two percoll layers and mononuclear cells from the serum/percoll interphase. Remaining erythrocytes were removed by hypotonic lysis. A second percoll gradient followed to remove cell debris. Purity of neutrophils was determined by cytopsin preparations and exceeded 95%, with few contaminating eosinophils, and <1% mononuclear cells, Viability of cells tested by Trypan blue exclusion was higher than 95%.

**Immunofluorescence analysis.** Isolated neutrophils, MNC, or B-cell lines were incubated with MoAb at 4°C. During incubation of effector cells with MoAb, polyclonal human IgG (4 mg/mL) was added to inhibit nonspecific binding to FcyRI. Cells were washed three times in phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA). FITC-labeled F(ab')2-fragments of goat antimouse or antihuman MoAb were used for staining. Cells were washed again and analyzed on a flow cytometer (EPICS Profile, Coulter). For each cell population, RFI was calculated as the ratio of
HLTII CLASS II AS TARGET ANTIGEN

Table 1. Reactivity of Malignant B-Cell Lines With Murine MoAbs

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Differentiation Stage</th>
<th>0-ALL</th>
<th>Burkitt’s Lymphoma</th>
<th>B-ALL</th>
<th>Mature B-Cell Lines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>REH</td>
<td>BJAB</td>
<td>RAJi</td>
<td>SB</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD19</td>
<td>J4.119</td>
<td>mlgGl</td>
<td>81</td>
<td>67</td>
<td>52</td>
</tr>
<tr>
<td>CD20</td>
<td>B1</td>
<td>mlgGl2a</td>
<td>1</td>
<td>nd</td>
<td>67</td>
</tr>
<tr>
<td>CD21</td>
<td>10B1a</td>
<td>mlgGl</td>
<td>1</td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>CD37</td>
<td>BL14</td>
<td>mlgGl</td>
<td>3</td>
<td>120</td>
<td>67</td>
</tr>
<tr>
<td>CD38</td>
<td>OKT10</td>
<td>mlgGl</td>
<td>nd</td>
<td>nd</td>
<td>29</td>
</tr>
<tr>
<td>CD40</td>
<td>5C3</td>
<td>mlgGl</td>
<td>nd</td>
<td>nd</td>
<td>21</td>
</tr>
<tr>
<td>HLA-DR/DP/DQ</td>
<td>CR3/43</td>
<td>mlgGl</td>
<td>198</td>
<td>nd</td>
<td>133</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>L243</td>
<td>mlgGl2a</td>
<td>111</td>
<td>72</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>F3.3</td>
<td>mlgGl</td>
<td>32</td>
<td>133</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>L227</td>
<td>mlgGl1</td>
<td>172</td>
<td>178</td>
<td>111</td>
</tr>
<tr>
<td>HLA-DR related</td>
<td>10D10</td>
<td>mlgGl2a</td>
<td>1</td>
<td>nd</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>LYM-1</td>
<td>mlgGl2a</td>
<td>1</td>
<td>nd</td>
<td>17</td>
</tr>
</tbody>
</table>

Results (from one of three experiments with similar results) are expressed as RFI, which was calculated for each cell line as the ratio of mean linear fluorescence intensity of relevant to irrelevant, isotype controlled antibodies.

mean linear fluorescence intensity of relevant to irrelevant, isotype-controlled antibodies.

**ADCC assays.** ADCC assays were performed as described. Briefly, target cells were labeled with 200 μCi 51Cr for 2 hours. After extensive washing with RF10', cells were adjusted to 10^6/mL. Effector cells (50 μL), sensitizing antibodies, and RF10' were added to round bottom microtiter plates. In some experiments blocking antibodies to Fcγ receptors were used at a final concentration of 50 μg/mL. Assays were started by adding the target cell suspension (50 μL) to each well, giving a final volume of 200 μL, and an effector to target (E:T) cell ratio of 40:1. After 4 hours at 37°C, assays were stopped by centrifugation, and 51Cr release from triplicates was measured in counts per minute (cpm). Percentage of cellular cytotoxicity was calculated using the formula:

\[ \text{% Specific Lysis} = \frac{(\text{Experimental cpm} - \text{Basal cpm})}{\text{Maximal cpm} - \text{Basal cpm}} \times 100 \]

with maximal 51Cr release determined by adding perchloric acid (3% final concentration) to target cells, and basal release measured in the presence of sensitizing antibodies without effector cells. Only low levels of antibody mediated, noncellular cytotoxicity (without neutrophil effector cells) and of antibody independent cytotoxicity (neutrophils without target antibodies) were observed under these assay conditions (<10% and <5%, respectively). Antibody independent killing was higher with MNC (see Table 2). For analysis of effects induced by Fc receptor antibodies, percent inhibition was calculated:

\[ \text{% Inhibition} = \frac{\text{\% Lysis Without Antibody} - \text{\% Lysis With Antibody}}{\text{\% Lysis Without Antibody}} \times 100 \]

Negative values determined by this formula are reported as percent stimulation in the presence of antibody.

Reverse cytotoxicity was tested using CR3 and FcγR antibody producing mouse hybridoma cells as targets. High membrane IgG expressing variants of hybridomas OKM-1 (CR3), 32.2 (FcγRI), 11.3 4 (FcγRII), and 3G8 (FcγRIII) were kindly provided by R. Graziano (Medarex). They were repeatedly reselected by cell sorting after staining with FITC-labeled rat antimouse IgG, resulting in RFI of 11.2, 9.0, 2.5, and 73.3 for hybridomas OKM-1, 32.2, IV.3 and 3G8, respectively. Reverse cytotoxicity assays against these hybridomas were performed in the absence of sensitizing antibodies, otherwise using the same conditions as above. In control experiments, rabbit polyclonal immune serum was used for sensitization antibodies, otherwise using the same conditions as above. In control experiments, rabbit polyclonal immune serum was used for sensitization antibodies, otherwise using the same conditions as above.

Table 2. ADCC Capacity of MNC and Neutrophils in ADCC Against RAJi Target Cells

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>MNC</th>
<th>HD PMN</th>
<th>G-CSF PMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Ab</td>
<td>—</td>
<td>11.7 ± 1.3</td>
<td>0.5 ± 0.2</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>CD19</td>
<td>murine</td>
<td>16.0 ± 1.6</td>
<td>1.2 ± 0.3</td>
<td>2.4 ± 0.8</td>
</tr>
<tr>
<td>CD37</td>
<td>chimeric</td>
<td>33.7 ± 5.3*</td>
<td>1.3 ± 0.6</td>
<td>0.6 ± 0.5</td>
</tr>
<tr>
<td>CD38</td>
<td>murine</td>
<td>16.4 ± 1.7</td>
<td>0.9 ± 0.6</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>HLA class II</td>
<td>murine</td>
<td>30.3 ± 2.6*</td>
<td>1.0 ± 0.4</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>chimeric</td>
<td>13.2 ± 5.3</td>
<td>1.2 ± 1.2</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>chimeric</td>
<td>31.8 ± 7.0t</td>
<td>0.9 ± 0.4</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

MNC were compared with PMN from healthy donors and with G-CSF primed neutrophils in ADCC with murine antibodies (all mlgGl) and with mouse/human chimeric FabFc13 constructs (all 2 μg/mL). Parental antibodies against B-cell related antigens were RFB9 (CD19), WR17 (CD37), and AT136 (CD38) (all mlgGl). Data are presented as mean ± SEM of at least five experiments. MNC killed RAJi target cells via MoAb F3.3 to HLA class II, but did not mediate ADCC with murine antibodies to other target antigens. Significant target cell lysis was obtained with MNC effector cells and chimeric constructs to all three B-cell antigens. PMN, in contrast, were consistently not able to lyse target cells with antibodies to B-cell antigens other than HLA class II.

Abbreviation: HD, healthy donors.

* P < .001.
† P < .05.
‡ P < .01.

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fector cell populations were not able to kill target cells without antigens. By unpaired (or when appropriate paired) Student's t-test. Levels of OKM-1 or with antibodies error of the mean (SEM). Differences between groups were analyzed only G-CSF primed neutrophils showed significant ADCC with MoAb CD20, CD21, CD37, CD38, CD40, and HLA class I variants of mIgG1 and mIgG2a to CD19 (CLB-CD19), and additional antibodies to CD19 (HD37, mlG1), CD21 (THB5, mlG2a), and CD37 (HD28, mlG2a) were tested under the same conditions as HLA class II antibodies. However, all of these antibodies consistently failed to induce cytotoxicity (<3% specific lysis) with G-CSF primed PMN or PMN from healthy donors.

Negative ADCC results with neutrophils and antibodies to classical B-cell antigens were not expected from published data with mononuclear effector cells. Therefore, control experiments comparing MNC and neutrophils in the presence of antibodies to HLA class II (F3.3), CD19 (RFB9), CD37 (WR17), and CD38 (AT13/5) were performed (Table 2). In these experiments, MNC, as well as unprimed and G-CSF primed PMN, mediated lysis of RAJI target cells with HLA class II antibody F3.3. Murine antibodies (all mlG1) to the other three target antigens mediated either no (PMN) or low levels of ADCC (MNC). Experiments with LAk cells have shown that antibodies with human Fc parts can mediate ADCC, although their parental murine versions proved ineffective in cellular cytotoxicity assays. Therefore, Fab(Fc)2 constructs with human Fc parts (see Materials and Methods) to CD19, CD37, and CD38 were tested. All these constructs bound strongly to RAJI cells, as shown by indirect immunofluorescence. However, only minimal killing was observed with these constructs at concentrations of 2 µg/mL, using neutrophils from patients during G-CSF treatment or neutrophils from healthy donors. MNC effector cells, however, mediated significant ADCC with all three constructs (Table 2). This cytotoxicity was completely blocked by F(ab')2 fragments (10 µg/mL) of FcγRIII antibody 3G8 (94.3% ± 3.2% inhibition), indicating that natural killer (NK) cells were the major effector cell population in these MNC preparations.

Expression of HLA-DR on RAJI cells is higher compared with other tested surface antigens (Table 1). Therefore, negative results with antibodies to these latter antigens could be due to insufficient target cell sensitization. In control experiments, a combination of antibodies to CD19, CD20, CD21, CD37, CD38, and CD40 was compared with HLA class II antibodies L243 or Lym-1. Immunofluorescence showed similar sensitization with the antibody combination and L243 (RFI 90 ± 111, respectively), but lower sensitization with Lym-1 (RFI 17). Cytotoxicity, however, was obtained with HLA class II antibodies L243 and Lym-1, but not with the combination of antibodies (Fig 2).

ADCC of neutrophils against various B-cell lines. In

![Fig 1. Comparison of MoAbs in ADCC against different B-cell antigens.](image-url)
experiments with RAJI target cells, HLA class II antibodies were shown to mediate ADCC effectively. Next, additional cell lines, representing different stages of B-cell maturation like 0-ALL (REH), Burkitt's lymphoma (BJAB), and mature B cells (BALL, SB, CESS, RM-1) were used as target cells in ADCC with neutrophil effector cells. Immunofluorescence with MoAb L243 showed all these B-cell lines to be positive for surface HLA-DR (see Table 1). PMN from healthy donors achieved modest levels of killing against all these target cell lines in the presence of MoAb L243. Similar results were obtained with HLA class II MoAb L227 (mlgG1), binding to a different epitope than L243, and with MoAb CR3/43 (mlgG1). In Fig 3, PMN from G-CSF receiving patients and from healthy donors were compared in MoAb L243 mediated cytotoxicity against four B-cell lines (REH, BALL, RAJI, RM-1). Neutrophils isolated during G-CSF treatment showed significantly enhanced tumor cell killing against all tested cell lines. There was no significant correlation between the level of HLA class II expression on different cell lines, and amount of lysis obtained with PMN isolated from patients during G-CSF therapy ($r = .18$, $P = .82$).

Experiments were performed to assess whether the negative results obtained with antibodies to B-cell related epitopes other than HLA class II were characteristic for RAJI cells. Therefore, additional B-cell lines (BJAB, BALL, RM-1, CESS and SB) were tested with murine antibodies to CD19, CD37, and HLA class II for neutrophil-mediated ADCC. As previously found, only MoAb to HLA class II consistently induced killing of target B cells with PMN, whereas results with MoAb J4.119 to CD19 and MoAb BL14 to CD37 were negative with all cell lines tested. Furthermore, Fab(FC)2 constructs specific for CD19, and CD37 were tested on CESS, BALL, and RM-1 target cell lines. Again, less than 5% specific lysis was observed.

*FcyR involvement in enhanced target cell killing of G-CSF primed neutrophils.* Neutrophils from healthy donors express the low-affinity Fcy receptors FcyRIIa (CD32) and FcyRIIb (CD16). During in vivo therapy with G-CSF, PMN additionally express the high-affinity FcyRI (CD64). To address the role of FcyRI as cytotoxic trigger molecule in ADCC, CR3 and Fcy receptor antibody producing hybridoma cell lines were tested as targets with PMN effectors (reverse ADCC). Hybridomas OKM-1 (CR3), 32.2 (FcyRI), IV.3 (FcyRII), and 3G8 (FcyRIII) were selected for high membrane expression of surface IgG (see Material and Methods). All three FcyR antibodies have been shown to trigger function of the respective receptors. In these reverse cytotoxicity assays, neutrophils from patients receiving G-CSF, but not PMN from healthy donors were able to lyse the anti-FcyRI hybridoma 32.2 (Fig 4). Both PMN from healthy donors and G-CSF treated patients showed similar levels of killing against the IV.3 hybridoma (anti-FcyRII), whereas the anti-CR3 (OKM-1) or the anti-FcyRIII (3G8) hybridomas were not killed by either effector cell population. The latter is probably due to the lack of a transmembrane region of the FcyRIIib molecule, which in contrast to FcyRIIa on monocytes and NK cells, is GPI-linked on PMN. In control experiments with a mononuclear effector cell population (containing NK cells and monocytes), the 3G8 hybridoma was also lysed. Both, 3G8 and OKM-1 hybridomas were killed by PMN in conventional ADCC with rabbit immune serum, excluding that they were generally

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**Fig 2. Role of target antigen in comparison with sensitization level for neutrophil-mediated ADCC.** G-CSF primed neutrophils were tested as effector cells in ADCC against RAJI target cells with HLA class II antibodies L243 and Lym-1 and with a mixture of MoAb against classical B-cell antigens CD19 (J4.119), CD20 (B1), CD21 (I0B1a), CD37 (I0B1), CD38 (AT13/5), and CD40 (5C3). Tumor cell sensitization measured by indirect immunofluorescence showed RFI of 17, 111, and 90 for Lym-1, L243 and the combination of MoAb (left panel). Significant ($P < .05$) ADCC was observed with MoAb L243 and Lym-1, but not with the mixture of classical B-cell antibodies (mean ± SEM, n = 3 experiments). Thus, insufficient target cell sensitization is not the explanation for the failure of B-cell antibodies to mediate ADCC with neutrophil effectors.

**Fig 3. ADCC of neutrophils against various B-cell lines.** Neutrophils from healthy donors (open symbols) and from G-CSF-treated patients (filled symbols) were compared as effector cells against various B-cell lines in the presence of HLA class II antibody L243. All experiments were performed as 4-hour $^{51}$Cr release assays at an ET ratio of 40:1. For reactivity of B-cell lines with MoAb L243, see Table 1. Each symbol represents results from one triplicate obtained from different donors. Means of each group are indicated by lines. Compared with PMN from healthy donors, in vivo primed neutrophils from G-CSF-treated patients showed significantly enhanced lysis against REH, BALL ($P < .01$, respectively), RAJI ($P < .001$), and RM-1 ($P < .05$).
CSF showed enhanced lysis of B cells using murine MoAb trigger molecules. Of mIgG1 and IgG2a isotypes. Dose response curves for FcyRI, MoAb IV.3 to FcyRII, or the combination of both. Blocking experiments were performed with MoAb 197 to gen density in FcyR mediated ADCC against B cells, resistant to PMN-mediated lysis. Thus, in vivo G-CSF primed neutrophils express FcyRI and FcyRII as cytotoxic trigger molecules.

Neutrophils isolated from patients during therapy with G-CSF showed enhanced lysis of B cells using murine MoAb of mIgG1 and IgG2a isotypes. Dose response curves for MoAb CR3/43 (mIgG1) and L243 (mIgG2a) were similar, and both showed half-maximal killing at 0.02 µg/mL (data not shown). To assess the role of antibody isotype and antigen density in FcyR mediated ADCC against B cells, blocking experiments were performed with MoAb 197 to FcyRI, MoAb IV.3 to FcyRII, or the combination of both. Lysis of RAJI target cells by PMN from healthy donors was almost completely inhibited by F(ab')2 fragments of MoAb 197 (CD16) did not significantly inhibit healthy donor or G-CSF primed PMN.

BsAb-mediated cytotoxicity of G-CSF primed neutrophils. We next evaluated whether BsAb with specificities for FcyR and HLA class II mediated cytotoxicity against malignant B cells. These BsAb were chemically cross-linked F(ab')2 containing F(ab')2 fragments of HLA class II MoAb F3.3, and F(ab')2 fragments from MoAb AT10 (CD32; FcyRII), or MoAb 22 (CD64; FcyRI), respectively. Thus, the specificities of the two derivate were: [HLA class II × FcyRII] and [HLA class II × FcyRI]. Binding of these BsAb to PMN from healthy donors, G-CSF-treated patients, and RAJI target cells was as expected from the parental antibodies. In the presence of MoAb F3.3 or of BsAb [HLA class II × FcyRII], both neutrophils from healthy donors and from G-CSF-treated patients lysed RAJI target cells (15.7 ± 0.1 and 11.0 ± 4.2 for healthy donors v 32.7 ± 14.2 and 33.9% ± 13.4% specific lysis at 10 µg/mL for G-CSF primed PMN, respectively, n = 3). In the presence of MoAb F3.3, BsAb [HLA class II × FcyRI] or [HLA class II × FcyRII], PMN from healthy donors were significantly (P < .05) less effective than neutrophils isolated during G-CSF treatment. Neutrophils from healthy donors did not lyse target cells via BsAb [HLA class II × FcyRI]. During G-CSF treatment, however, PMN killed RAJI targets via BsAb [HLA class II × FcyRI] very effectively (Fig 6). With G-CSF primed PMN, the killing via [HLA class II × FcyRII] BsAb was significantly (P < .05 at 2 µg/mL) higher than killing via MoAb F3.3, or BsAb [HLA class II × FcyRII], respectively.

Comparing MNC and G-CSF primed neutrophils in BsAb-mediated cytotoxicity. Among the different B-cell antigens, CD37 is a promising candidate for immunotherapy because

**Fig 4.** FcyR as cytotoxic trigger molecules on PMN. Hybridomas, selected for high surface expression of antibodies to CR3 (OKM-1), FcyRI (32.2), FcyRII (IV.3), and FcyRIII (3G8) were used as targets in reverse ADCC mediated by PMN from healthy donors or from G-CSF-treated patients (G-CSF). Results from n = 6 (FcyRI and CR3), or n = 12 (FcyRII and FcyRI) pairs of donors are presented as mean ± SEM (indicated by error bars). PMN from control donors and from patients during G-CSF treatment differed significantly (P < .001) in their capacity to lyse the anti-FcyRI hybridoma, killed the anti-FcyRII hybridoma with similar efficacy, and were both unable to lyse the anti-FcyRIII and the anti-CR3 hybridomas in reverse ADCC.

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BsAb-mediated cytotoxicity of G-CSF primed neutrophils. We next evaluated whether BsAb with specificities for FcyR and HLA class II mediated cytotoxicity against malignant B cells. These BsAb were chemically cross-linked F(ab')2 containing F(ab')2 fragments of HLA class II MoAb F3.3, and F(ab')2 fragments from MoAb AT10 (CD32; FcyRII), or MoAb 22 (CD64; FcyRI), respectively. Thus, the specificities of the two derivate were: [HLA class II × FcyRII] and [HLA class II × FcyRI]. Binding of these BsAb to PMN from healthy donors, G-CSF-treated patients, and RAJI target cells was as expected from the parental antibodies. In the presence of MoAb F3.3 or of BsAb [HLA class II × FcyRII], both neutrophils from healthy donors and from G-CSF-treated patients lysed RAJI target cells (15.7 ± 0.1 and 11.0 ± 4.2 for healthy donors v 32.7 ± 14.2 and 33.9% ± 13.4% specific lysis at 10 µg/mL for G-CSF primed PMN, respectively, n = 3). In the presence of MoAb F3.3, BsAb [HLA class II × FcyRI] or [HLA class II × FcyRII], PMN from healthy donors were significantly (P < .05) less effective than neutrophils isolated during G-CSF treatment. Neutrophils from healthy donors did not lyse target cells via BsAb [HLA class II × FcyRI]. During G-CSF treatment, however, PMN killed RAJI targets via BsAb [HLA class II × FcyRI] very effectively (Fig 6). With G-CSF primed PMN, the killing via [HLA class II × FcyRII] BsAb was significantly (P < .05 at 2 µg/mL) higher than killing via MoAb F3.3, or BsAb [HLA class II × FcyRII], respectively.

Comparing MNC and G-CSF primed neutrophils in BsAb-mediated cytotoxicity. Among the different B-cell antigens, CD37 is a promising candidate for immunotherapy because

**Fig 4.** FcyR as cytotoxic trigger molecules on PMN. Hybridomas, selected for high surface expression of antibodies to CR3 (OKM-1), FcyRI (32.2), FcyRII (IV.3), and FcyRIII (3G8) were used as targets in reverse ADCC mediated by PMN from healthy donors or from G-CSF-treated patients (G-CSF). Results from n = 6 (FcyRI and CR3), or n = 12 (FcyRII and FcyRI) pairs of donors are presented as mean ± SEM (indicated by error bars). PMN from control donors and from patients during G-CSF treatment differed significantly (P < .001) in their capacity to lyse the anti-FcyRI hybridoma, killed the anti-FcyRII hybridoma with similar efficacy, and were both unable to lyse the anti-FcyRIII and the anti-CR3 hybridomas in reverse ADCC.

resistant to PMN-mediated lysis. Thus, in vivo G-CSF primed neutrophils express FcyRI and FcyRII as cytotoxic trigger molecules.

Neutrophils isolated from patients during therapy with G-CSF showed enhanced lysis of B cells using murine MoAb of mIgG1 and IgG2a isotypes. Dose response curves for MoAb CR3/43 (mIgG1) and L243 (mIgG2a) were similar, and both showed half-maximal killing at 0.02 µg/mL (data not shown). To assess the role of antibody isotype and antigen density in FcyR mediated ADCC against B cells, blocking experiments were performed with MoAb 197 to FcyRI, MoAb IV.3 to FcyRII, or the combination of both. Lysis of RAJI target cells by PMN from healthy donors was almost completely inhibited by F(ab')2 fragments of MoAb 197 (CD16) did not significantly inhibit healthy donor or G-CSF primed PMN.

BsAb-mediated cytotoxicity of G-CSF primed neutrophils. We next evaluated whether BsAb with specificities for FcyR and HLA class II mediated cytotoxicity against malignant B cells. These BsAb were chemically cross-linked F(ab')2 containing F(ab')2 fragments of HLA class II MoAb F3.3, and F(ab')2 fragments from MoAb AT10 (CD32; FcyRII), or MoAb 22 (CD64; FcyRI), respectively. Thus, the specificities of the two derivate were: [HLA class II × FcyRII] and [HLA class II × FcyRI]. Binding of these BsAb to PMN from healthy donors, G-CSF-treated patients, and RAJI target cells was as expected from the parental antibodies. In the presence of MoAb F3.3 or of BsAb [HLA class II × FcyRII], both neutrophils from healthy donors and from G-CSF-treated patients lysed RAJI target cells (15.7 ± 0.1 and 11.0 ± 4.2 for healthy donors v 32.7 ± 14.2 and 33.9% ± 13.4% specific lysis at 10 µg/mL for G-CSF primed PMN, respectively, n = 3). In the presence of MoAb F3.3, BsAb [HLA class II × FcyRI] or [HLA class II × FcyRII], PMN from healthy donors were significantly (P < .05) less effective than neutrophils isolated during G-CSF treatment. Neutrophils from healthy donors did not lyse target cells via BsAb [HLA class II × FcyRI]. During G-CSF treatment, however, PMN killed RAJI targets via BsAb [HLA class II × FcyRI] very effectively (Fig 6). With G-CSF primed PMN, the killing via [HLA class II × FcyRII] BsAb was significantly (P < .05 at 2 µg/mL) higher than killing via MoAb F3.3, or BsAb [HLA class II × FcyRII], respectively.

Comparing MNC and G-CSF primed neutrophils in BsAb-mediated cytotoxicity. Among the different B-cell antigens, CD37 is a promising candidate for immunotherapy because
HLA CLASS II AS TARGET ANTIGEN

it is expressed at high levels on a broad spectrum of malignant B cells and is not shed from the surface of target cells. To assess whether lysis of malignant B cells by G-CSF primed PMN via MoAb to CD37 could be improved using HLA class III antibodies, G-CSF primed PMN may constitute an interesting effector cell population for immunotherapy of B-cell malignancies.

DISCUSSION

The data presented show activated neutrophils to be capable of killing a broad spectrum of malignant B-cell lines in the presence of HLA class II antibodies. Although HLA class II antibodies differed in their efficacy to mediate ADCC, all six antibodies tested showed target cell lysis with G-CSF primed effector cells. Remarkably, our results with antibodies to potential target antigens like CD19, CD20, CD21, CD37, CD38, and CD40 and activated neutrophil effector cells were negative. This was in contrast to data with MNC, which mediated ADCC with murine antibodies to HLA class II and with chimeric constructs to CD19, CD37, and CD38. Thus, target antigen selection for immunotherapy with antibodies may be influenced by the desired effector cell population, which may be increased in number and function by combination with cytokines (eg, G-CSF).

Results with neutrophils and HLA class II antibodies of mlgG1 or mlgG2a isotype, with chimeric antibodies against CD19, CD37, and CD38 and with bispecific antibodies (CD37 × FcyRII versus [HLA class II × FcyRI]), exclude insufficient interaction of murine MoAb with neutrophil Fc receptors as a possible explanation for negative results with antibodies to classical B-cell related antigens. Expression levels of HLA class II on malignant B cells are often higher than those of other B-cell antigens (see Table 1). However, differences in target cell sensitization could not explain results in ADCC because (1) a combination of MoAbs to B-cell antigens, which showed similar staining intensity to that of a single HLA class II antibody, did not mediate lysis, (2) no correlation between HLA-DR expression and susceptibility to neutrophil-mediated lysis was found between different B-cell lines, (3) target cell lysis was observed at very low, sub saturating concentrations of HLA class II antibodies, and (4) antibodies to HLA class II related molecules like 1D10 and Ly-1, which showed lower levels of sensitization than antibodies to some of the classical B-cell related epitopes, mediated ADCC with G-CSF primed neutrophils.

HLA class II antibodies have been shown to have antiproliferative effects on malignant B cells. Similar effects have been described for CD40 and anti-μ antibodies. However, the latter two did not induce killing of RAJI tar-
gets, arguing against a selective effect of neutrophils on nondividing B cells. Neutrophil-mediated ADCC is an active process of specialized effector cells because (1) none of the tested HLA class II antibodies induced significant 51Cr-release under the conditions of our assay in the absence of neutrophils, (2) no antibody-independent cytotoxicity was observed with neutrophil effector cells, (3) cross-linking of antibody-coated target antigens by Fc receptor expressing KG-1 cells did not induce ADCC of tumor target cells (data not shown), and (4) no lysis of unsensitized bystander cells occurred.

Modulation of target antigens on malignant B cells is variable depending, eg, on the antigen itself, the isotype of antibodies,29 and the presence of Fc receptor positive bystander cells.31 A comparative analysis of different antigens showed HLA-DR to be internalized slowly, but similar results were also obtained with a CD20 antibody.42 Many of the B-cell related antigens,43 and also HLA-DR,44 were shown to induce intracellular signaling and activation of B cells after cross-linking with MoAbs. For example, HLA class II antibodies were shown to induce tumor necrosis factor (TNF) production by malignant B cells,45 which is a potent activator of PMN-mediated cytotoxicity.31 In control experiments, blocking antibodies to TNF, however, had no effect on unstimulated ADCC, although the effect of exogenous TNF could be completely blocked by TNF antibodies (data not shown). Other explanations include antibody-induced expression of molecules such as adhesion molecules (which may render the malignant cells more sensitive to lysis) and the different capacity of various B-cell related antigens and HLA-DR to cluster on the target cell surface. The influence of these factors for neutrophil-mediated ADCC is currently under investigation.

Several studies have suggested an active role for neutrophils in the immunosurveillance against malignant tumors.5,46-48 During therapy with G-CSF, significantly enhanced in vitro cytotoxicity of isolated PMN against glioblastoma, squamous cell, ovarian,10 and breast carcinoma cell lines (manuscript in preparation) was observed with sensitizing MoAb to the oncogene products EGF-R and HER-2/neu. G-CSF is known to enhance or to prime different cellular functions of neutrophils like phagocytosis, degranulation, and production of oxygen radicals.49 Most importantly for ADCC, neutrophils during G-CSF therapy express high levels of the high-affinity FcγRI (CD64).5,10 The causal role of G-CSF versus other patient characteristics (eg, underlying disease, other treatments) for these phenotypical changes is indicated by healthy volunteer studies with G-CSF,11 and by investigating patients serially (before, during, and after G-CSF).9,46 FcγRI is an effective cytotoxic trigger molecule on G-CSF primed PMN, as demonstrated in reverse cytotoxicity assays against FcγR receptor antibody producing hybridoma targets, with blocking antibodies to Fcγ receptors and with bispecific antibodies with one specificity for FcγRI. Physiologically, FcγRI is expressed on monocytes and macrophages and at low numbers on neutrophils. This restricted expression on potential cytotoxic effector cells renders FcγRI an interesting molecule for a therapeutic approach with BsAb.12 A recently completed phase I study with MDX210, an [HER-2/neu × FcγRII] BsAb, in patients with breast and ovarian cancer showed biological and clinical activity at well tolerated doses.13 A combination of the same BsAb and G-CSF is currently being tested in a phase I study in patients with stage IV breast cancer at our institution. BsAb doses as low as 0.35 mg/m² induced a systemic response as indicated by measurable serum levels of interleukin (IL)-6 and TNF-α shortly after BsAb application.49

Leukemias and lymphomas have been targeted with MoAb in several clinical trials. With mononuclear effector cells, several of these antibodies were shown to mediate ADCC, especially when chimeric constructs were used (Table 2). In this paper, G-CSF primed neutrophils were shown to be potent effector cells against malignant B-cell lines only in the presence of HLA class II antibodies. With antibodies to other classical B-cell related antigens, however, neutrophils proved completely ineffective. This suggests that requirements for target antigens may differ between potential effector cell populations for immunotherapy. Although, HLA class II expression is not B-cell specific, several lines of evidence suggest a role for HLA class II antibodies in immunotherapy of malignant B cells. In a syngeneic B-cell tumor model, antibodies to HLA-DR could cure and prolong survival in a considerable percentage of mice.50 Under certain experimental conditions, immune suppressive effects can be achieved with HLA-DR antibodies. However, anti-HLA-DR treatment was well tolerated by mice, and no compromise of immune functions was observed in long-term surviving animals.51 In the presence of HLA class II MoAb Lym-1, freshly obtained CLL cells from patients were lysed by IFN-γ-activated neutrophils.52 Lym-1 was subsequently tested in patients with B-cell malignancies. Results with the unconjugated antibody showed minimal toxicity and some clinical responses.50 Radioconjugates of Lym-1 were clinically more effective, but were considerably myelosuppressive.51 MoAb 1D10, recognizing an HLA class II related epitope, was shown to preferentially bind to malignant B cells,53 and a clinical trial with a humanized 1D10 is starting soon (G.W.). In animal studies, certain BsAb were more effective than their parental MoAb in curing mice from their lymphomas.52,53 Our in vitro data suggest an approach with an [HLA class II × FcγRI] BsAb in combination with G-CSF in patients with malignant B-cell disorders. To further evaluate efficacy and potential toxicity of this approach in vivo, we are currently establishing syngeneic lymphoma models in human FcγRI transgenic mice.

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HLA class II as potential target antigen on malignant B cells for therapy with bispecific antibodies in combination with granulocyte colony-stimulating factor

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