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Triggering FCα-Receptor I (CD89) Recruits Neutrophils as Effector Cells for CD20-Directed Antibody Therapy[1]

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CD20 Abs induce clinical responses in lymphoma patients, but there are considerable differences between individual patients. In ^{51}Cr release assays with whole blood as effector source, RAJI cells were effectively killed by a mouse/human chimeric IgG1 construct of CD20 Ab 1F5, whereas ARH-77 proved resistant to killing by this Ab. When whole blood was fractionated into plasma, mononuclear cells, or granulocytic effector cells, RAJI cells were effectively killed in the presence of complement-containing plasma, whereas the mature B cell line ARH-77 proved complement resistant. However, with a bispecific Ab (BsAb) against the myeloid receptor for IgA (CD89; FcαRI) and CD20, a broad range of B cell lines were effectively killed. FcαRI is expressed on monocytes/macrophages, neutrophils, and eosinophils. As the numbers of these effector cells and their functional activity can be enhanced by application of G-CSF or GM-CSF, lysis via (FcαRI × CD20) BsAb was significantly enhanced in blood from patients during therapy with these myeloid growth factors. Interestingly, the major effector cell population for this BsAb were polymorphonuclear neutrophils, which proved ineffective in killing malignant B cells with murine, chimeric IgG1, or FcγRI- or FcγRIII-directed BsAbs against CD20. Experiments with blood from human FcαRI/FcγRI double-transgenic mice showed corresponding results, allowing the establishment of relevant syngenic animal models in these mice. In conclusion, the combination of myeloid growth factors and an (FcαRI × CD20) BsAb may represent a promising approach to improve effector cell recruitment for CD20-directed lymphoma therapy. *The Journal of Immunology,* **2000, 165: 5954–5961.**

Malignant lymphomas have been increasing in incidence over the last two decades and are the most common neoplasm of young adults (1). In Western countries, ~85% are of B cell origin, and most patients with low or intermediate grade lymphomas, or relapses of high grade lymphomas, have a poor prognosis despite major advances in chemo- and radiotherapy, including bone marrow transplantation (2). Since the description of the hybridoma technology by Köhler and Milstein (3), more than two decades passed before the mouse/human chimeric CD20 Ab C2B8 was approved by the Food and Drug Administration as the first mAb for treatment in oncology. The CD20 Ag seemed to be a particularly promising target for immunotherapy of B cell neoplasms (4) because it is expressed on the cell surface of >90% of malignant B cells but not on hemopoietic stem cells, normal plasma cells, myeloid, T lineage, endothelial, or other nonlymphoid cells (5). Upon binding of Abs, CD20 does not significantly modulate or shed, and a plethora of potential effector mechanisms of mAbs were shown to be recruited, such as Ab-dependent cell-mediated cytotoxicity (ADCC)[3] by mononuclear effector cells, complement-dependent lysis, initiation of intracellular signals such as calcium fluxes, inhibition of cell growth, and induction of cell differentiation. Importantly, CD20 Abs were shown to induce apoptosis of malignant B cell lines, especially after intensive cross-linking, e.g., by receptors for the Fc domain of IgG (FcγR)-expressing cells (6).

To recruit cell-mediated effector mechanisms, Abs must interact with Ig FcRs, which are divided into Fcα-, Fcϵ-, or FcγRs, depending on their specificity for IgA, IgE, or IgG, respectively (7). FcγR isoforms are grouped into two classes of low affinity receptors named FcγRII (CD32) and FcγRIII (CD16) and a single high affinity class, FcγRI (CD64) (8). A pivotal role for FcRs as mediators of therapeutic Ab effects in vivo was suggested by studies in mice in which the signaling machinery of FcRs was disrupted by gene targeting of the FcR common γ-chain (9). In contrast to their littermates, these genetically modified animals were no longer protected from tumor growth by therapeutic mAbs. Among other potential FcR-mediated functions for therapeutic Abs, such as phagocytosis of tumor cells (10) and, subsequently, enhanced presentation of tumor Ags to T cells (11) or improved induction of apoptosis by target Ag cross-linking (6), ADCC is considered important in vivo (12). The capacity to mediate ADCC has been demonstrated in vitro for monocytes/macrophages, NK cells, as well as eosinophilic and neutrophilic granulocytes. Neutrophils are increasingly recognized as an important effector cell population for growth arrest and rejection of malignant tumors in vivo (13). In

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[3] Abbreviations used in this paper: ADCC, Ab-dependent cell-mediated cytotoxicity; BsAb, bispecific Ab; FcαRI, myeloid Fc receptor for IgA; FcγR, receptors for the Fc domain of IgG; PMNs, polymorphonuclear neutrophils; MNC, mononuclear cell; RFI, relative fluorescence intensity; BALL, B acute lymphoblastic leukemia; SH, sulfhydryl; *o*-PDM, *o*-phenylenedimaleimide; SNARF, seminapthorhodafluor.

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vitro, polymorphonuclear neutrophils (PMNs) were the predominant effector cell population for the killing of breast cancer cells in the presence of HER-2/neu Abs, especially after preactivation of neutrophils by G-CSF (14), which is known to induce expression of FcγRI as an additional cytotoxic trigger molecule (15, 16). However, when we analyzed the capacity of neutrophils to kill malignant B cells, we observed that they were very efficient in killing B cells with Abs directed against HLA class II but proved completely ineffective with Abs to other B cell-associated Ags such as CD19, CD20, CD21, CD37, or CD38 (17, 18).

Bispecific Abs (BsAbs), containing one specificity against a tumor target Ag and another specificity against select epitopes of an activating FcR on cytotoxic cells, are an elegant way to improve effector cell recruitment for Ab therapy (19, 20). Recently, we demonstrated that, in addition to the IgG receptors FcγRI (CD64) and FcγRIII (CD16), the myeloid receptor for IgA (FcαRI, CD89) is an interesting trigger molecule for BsAb therapy (21). FcαRI is constitutively expressed on monocytes/macrophages, eosinophils, neutrophils, and some types of dendritic cells, but importantly it is not found on noneffector cell populations (22). Activation of FcαRI was shown to trigger phagocytosis, respiratory burst, cytokine release, and ADCC. As some otherwise resistant solid tumor cell lines were effectively killed by growth factor-primed PMNs in the presence of FcαRI-directed BsAbs, we were interested to test whether the Ag restriction of neutrophils in killing malignant B cells could be overcome by targeting FcαRI instead of FcγRs. As described in this manuscript, PMNs were indeed found to effectively lyse malignant B cells with an (FcαRI $\times$ CD20) BsAb, but were again unable to kill CD20-positive B cells with IgG or FcγR-directed BsAbs against the CD20 target Ag. These results demonstrate for the first time that the combination of myeloid growth factors and (FcαRI $\times$ CD20) BsAb may significantly improve effector cell recruitment for CD20-directed immunotherapy.

Materials and Methods

Blood donors

Experiments reported here were approved by the Ethical Committee of the University of Erlangen-Nürnberg (Erlangen, Germany), in accordance with the Declaration of Helsinki. After informed consent, 10–20 ml of peripheral blood was drawn from healthy volunteers or from patients receiving rhG-CSF (3–5 μg/kg of body weight, Neupogen; Hoffmann-LaRoche, Basel, Switzerland) or rhGM-CSF (5 μg/kg of body weight, Leukomax; Essex Pharma, Munich, Germany) based on clinical indications. For analysis during growth factor treatment, patients had at least 3 days of cytokine therapy. Relative fluorescence intensities (RFIs) for expression of FcαRI (CD89) or FcγRI (CD64) on PMNs from healthy donors or from patients treated with G-CSF or GM-CSF are presented in Table I. Staining for FcαRI was significantly higher than that for FcγRI on healthy donor- and GM-CSF-primed PMNs, but not on G-CSF-primed PMNs. Expression of FcγRI was significantly higher on G-CSF-primed PMNs compared with healthy donor- or GM-CSF-primed neutrophils.

Table I. *Expression of FcγRI and FcαRI on PMN from healthy donors, G-CSF-, or GM-CSF-treated patients*

	Healthy Donor	G-CSF	GM-CSF
RFI for FcγRI	1.3 $\pm$ 0.07	9.5 $\pm$ 1.3[a]	1.5 $\pm$ 0.2
Number of experiments	16	9	8
RFI for FcαRI	9.9 $\pm$ 1.7	12.5 $\pm$ 1.0	5.9 $\pm$ 1.9
Number of experiments	16	9	8
p value for comparison between FcαRI and FcγRI	$<$0.05	0.09	$<$0.05

[a] PMN from G-CSF-treated patients expressed significantly higher levels of FcγRI than neutrophils from healthy donors, or from GM-CSF-treated patients, whereas expression of FcαRI was not significantly different between the three groups.

Human FcαRI/FcγRI double-transgenic mice

FcαRI/FcγRI double-transgenic mice were generated by crossing human FcγRI- with human FcαRI-transgenic mice. Human FcγRI-transgenic mice were generated by injection of an 18-kb human genomic DNA fragment carrying the *FcγRIA* gene into FVB/N oocytes (23). A 41-kb cosmid clone containing the human *FcαRI* gene was used as a construct to generate FcαRI transgenic mice (24). Expression of transgenes was checked by flow cytometry of peripheral blood cells, using FITC-labeled anti-FcγRI mAb 22 or anti-FcαRI mAb A77 (both obtained from Medarex, Annendale, NJ), respectively. All mice were bred at the Transgenic Mouse Facility of Utrecht University (Utrecht, The Netherlands). To induce neutrophil FcγRI expression and to increase blood neutrophil counts, mice were s.c. injected with murine G-CSF for 4 days (1.6 μg/mouse/day) before ADCC experiments. Murine G-CSF was provided by Dr. J. Andresen (Amgen, Thousand Oaks, CA).

Cell lines

The malignant B cell lines B acute lymphoblastic leukemia (BALL), RAJI (Burkitt's lymphoma), ARH-77, and CESS (both mature B cell lines) were obtained from the American Type Culture Collection (Manassas, VA). RM-1 (EBV-transformed B cell line) and BJAB (Burkitt's lymphoma) were obtained from Dr. G. Bonnard (National Cancer Institute, Bethesda, MD) and Dr. W. Leibold (Department of Veterinary Medicine, University of Hannover, Hannover, Germany), respectively. All cells were kept in $RF10^+$ medium consisting of RPMI 1640 (Life Technologies, Paisley, U.K.) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 U/ml streptomycin, and 4 mmol/L L-glutamine (all obtained from Life Technologies).

mAbs and Ab constructs

Abs F3.3 (HLA class II, mIgG1), AT80 (CD20, mIgG1), and H147 (CD20, mIgG3) were generated, and WR17 (CD37, mIgG1; Ref. 25) and 1F5 (CD20, mIgG2a; Ref. 26) were produced from the original hybridomas at the Tenovus Research Laboratory (University of Southampton, Southampton, U.K.). CD20 Ab MEM-97 (mIgG1) was provided by Dr. V. Horejsi (Institute for Molecular Genetics, Prague, Czech Republic). B1 (mIgG2a), Leu-16 (mIgG1), and mouse/human chimeric C2B8 (hIgG1) were purchased from Coulter (Hialeah, FL), Dianova (Hamburg, Germany), and Hoffmann-LaRoche, respectively. Abs A77 (FcαRI, CD89; mIgG1), 22 (FcγRI, CD64; mIgG1), 3G8 (FcγRIII, CD16; mIgG1), 4G7 (CD19, mIgG1), FITC-labeled A77 or 22, and 14.1 (a novel fully human IgG1 Ab against CD89) were obtained from Medarex. PE-labeled Gr-1 Ab was obtained from PharMingen (San Diego, CA).

BsAbs (FcαRI $\times$ CD19), (FcαRI $\times$ CD20), (FcαRI $\times$ CD37), (FcαRI $\times$ HLA class II), (FcγRI $\times$ CD20), and (FcγRIII $\times$ CD20) were produced by chemically cross-linking F(ab′) fragments of target Ag Ab 4G7 (CD19), 1F5 (CD20), WR17 (CD37), or F3.3 (HLA class II) with trigger molecule Ab A77 (FcαRI, CD89), 22 (FcγRI, CD64), or 3G8 (FcγRIII, CD16) as described (27). Additional (FcαRI $\times$ CD20) BsAbs were generated using CD89 Abs A77 or 14.1, and CD20 Abs AT80 or C2B8. Briefly, $F(ab'\gamma)_2$ fragments were produced by limited proteolysis with pepsin and were then reduced with mercaptoethanol amine to provide Fab′γ with free hinge-region sulfhydryl (SH) groups. The SH groups on one of the Fab′γ(SH) partners were then fully alkylated with excess *o*-phenylenedimaleimide (*o*-PDM) to provide free maleimide groups (mal). Finally, the two preparations Fab′γ(mal) and Fab′γ(SH) were combined at a ratio of 1:1 to generate heterodimeric constructs. After purification by size exclusion chromatography and characterization by HPLC, samples were sterilized by filtration and stored at 4°C.

The chimeric $Fab(Fc)_2$ construct of CD20 mAb 1F5 (ch1F5), consisting of a single Fab′ fragment from mouse Ab 1F5 chemically conjugated to two human Fc fragments, was prepared as reported (28). Briefly, F(ab′)-*o*-PDM of 1F5 were produced as described above. To prepare human Fcγ, human serum IgG was digested with papain, and resulting Fcγ fragments were separated and purified. Following reduction of Fcγ fragments, fragments were incubated with F(ab′)-*o*-PDM to yield $Fab(Fc)_2$ constructs with mainly human IgG1 Fc fragments.

Serial dilutions of Ab derivatives were analyzed for binding to effector and target cells by indirect immunofluorescence. Half-maximal binding to tumor cells occurred at 0.06, 0.05, and 0.09 μM for the three ((22 $\times$ 1F5), (3G8 $\times$ 1F5), or (A77 $\times$ 1F5)) BsAbs, respectively, and at 0.5 μM for the chimeric 1F5 Ab. Similarly, half-maximal binding to effector cells was determined at 0.012, 0.1, and 0.025 μM for the three BsAbs, respectively.

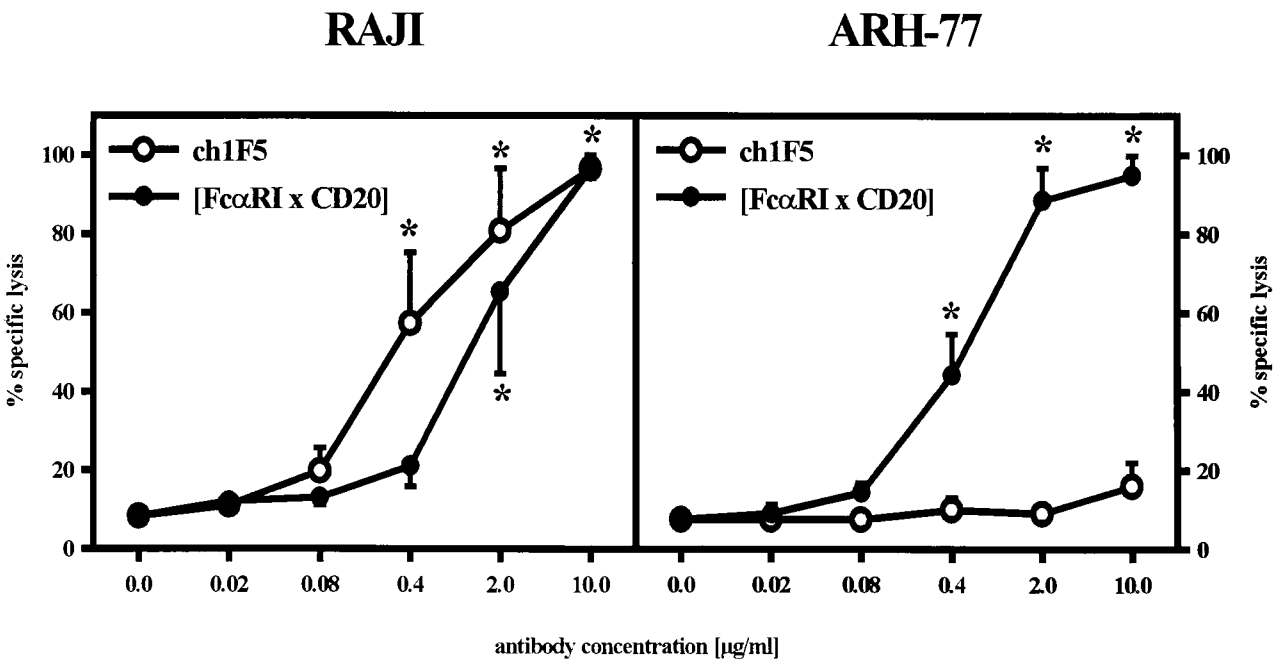

FIGURE 1. Improved killing of the mature B cell line ARH-77 by (FcαRI × CD20) BsAb. Blood from three GM-CSF-treated patients was analyzed in whole blood ADCC against RAJI (Burkitt's lymphoma) or ARH-77 (mature B) cell lines comparing chimeric CD20 Ab 1F5 (ch1F5) with the respective FcαRI-directed BsAb (FcαRI × CD20). Both constructs mediated effective lysis of RAJI cells. However, only the BsAb, but not the chimeric Ab was effective against ARH-77 cells. Results are presented as mean ± SEM, with significant lysis indicated by ∗.

Avidity of parental mAbs 22 (FcγRI) and A77 (FcαRI) to isolated PMNs from G-CSF-treated patients was 0.6 and 10 nM, respectively.

Isolation of mononuclear cells (MNCs) and neutrophil effector cells

Neutrophils were isolated by a method slightly modified from that described in (15). Briefly, citrate-anticoagulated blood was layered over a discontinuous Percoll (Seromed, Berlin, Germany) gradient consisting of 70 and 62% Percoll. After centrifugation, neutrophils were collected at the interphase between the two Percoll layers, and MNCs from the serum/Percoll interphase. Remaining erythrocytes were removed by hypotonic lysis. Purity of neutrophils was determined by cytospin preparations and exceeded 95%, with few contaminating eosinophils in healthy donors and G-CSF-treated patients and up to 25% eosinophils in preparations from GM-CSF-treated patients. Viability of cells tested by trypan blue exclusion was >95%. MNC contamination was <1% in all preparations.

Immunofluorescence analysis

For indirect immunofluorescence, polyclonal human IgG (4 mg/ml) was added to inhibit nonspecific binding to FcγRI. Cells were washed three times in PBS supplemented with 1% BSA. FITC-labeled $F(ab')_2$ fragments of goat anti-mouse or anti-human mAbs 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 mean linear fluorescence intensity of relevant to irrelevant, isotype-controlled Abs.

ADCC assays

ADCC assays were performed as described (14). Briefly, target cells were labeled with 200 μCi ^{51}Cr for 2 h. After extensive washing with $RF10^+$, cells were adjusted to 10^5/ml. Whole blood or isolated effector cells (50 μl), sensitizing Abs, and $RF10^+$ were added to round-bottom microtiter plates. Assays were started by adding the target cell suspension (50 μl), giving a final volume of 200 μl, and an E:T cell ratio of 40:1 with isolated human effector cells. After 3 hours at 37°C, assays were stopped by centrifugation, and ^{51}Cr release from triplicate samples was measured in cpm. Percentage of cellular cytotoxicity was calculated using the formula: % specific lysis = (experimental cpm − basal cpm)/(maximal cpm − basal cpm) × 100, with maximal ^{51}Cr release determined by adding perchloric acid (3% final concentration) to target cells, and basal release was measured in the absence of sensitizing Abs and effector cells. Ab-independent cytotoxicity (effectors without target Abs) was observed in whole blood assays and with mononuclear effector cells, but not with PMNs. ADCC experiments with murine whole blood were performed in duplicate.

Calcium mobilization assay

Intracellular free calcium levels were analyzed by a flow cytometry assay (29). Whole blood from G-CSF-treated human FcαRI/FcγRI double-transgenic mice was incubated with 0.2 × PBS for 1 min to lyse erythrocytes. White blood cells were then incubated with seminapthorhodafluor (SNARF)-1 (2.8 μM) and Fluo-3 (1.4 μM) (Molecular Probes, Eugene, OR) for 30 min at 37°C. After washing, cells were incubated with anti-CD64 mAb 22 (10 μg/ml) or anti-CD89 mAb A77 (10 μg/ml) for 30 min at room temperature, washed twice, and resuspended in calcium mobilization buffer at a concentration of 1×10^7 cells/ml. PMNs were identified by forward and side scatter profiles, and cells were measured at a rate of ≈140 cells/s. The first 24 s of each run were used to establish baseline intracellular calcium levels, after which cross-linking goat anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL) was added at concentrations ranging from 1 to 10 μg/ml. $[Ca^{2+}]_i$ baseline levels were subtracted from all measurements, and % Fluo-3/SNARF-1 ratio was calculated by dividing the Fluo-3/SNARF-1 ratio at a given time point by the maximal Fluo-3/SNARF-1 ratio of each individual experiment × 100%.

Statistical analysis

Group data are reported as mean ± SEM. Differences between groups were analyzed by unpaired (or, when appropriate, paired) Student's *t* tests. Significance was accepted when $p < 0.05$.

Results

Comparison of human IgG1 chimeric and FcαRI-directed BsAbs against CD20

Chimeric IgG1 Abs against CD20 are particularly effective in follicular or post transplant lymphoma patients, whereas results in patients with other histologies, such as mantle cell lymphoma or diffuse large cell lymphoma, are less impressive (30). We established whole blood cytotoxicity assays against two prototypic CD20-positive B cell lines: RAJI, a Burkitt's lymphoma, which proved sensitive for anti-CD20-mediated killing, and ARH-77, a mature B cell line, which was not lysed by the chimeric CD20 Ab

1F5 (Fig. 1). Effector mechanisms operative in the killing of RAJI cells were analyzed by fractionating whole blood into plasma, MNCs, and granulocytes. Thus, we found chimeric Ab-mediated killing of RAJI cells by plasma (43 ± 12%) and MNCs (45 ± 16%) but not by granulocytes (5 ± 5%, $n = 4$), the latter in agreement with our previous observation that neutrophils do not lyse malignant B cells in the presence of Abs to "classical" B cell Ags (17, 18). Plasma-mediated lysis of RAJI cells by chimeric 1F5 was completely abolished by heat inactivation of plasma (56°C for 30 min), suggesting that complement-dependent cytotoxicity was the underlying mechanism. As expected, ARH-77 cells were not killed by any of these fractions in the presence of the chimeric CD20 Ab. However, interestingly, both cell lines were effectively lysed by whole blood from GM-CSF-treated patients using an (FcαRI × CD20) BsAb (Fig. 1). Analysis of the lytic fraction in whole blood identified PMNs as the major effector population for this BsAb (68 ± 8, 16 ± 3, and 3 ± 1% specific lysis for PMNs, MNCs, or plasma, respectively; $n = 5$).

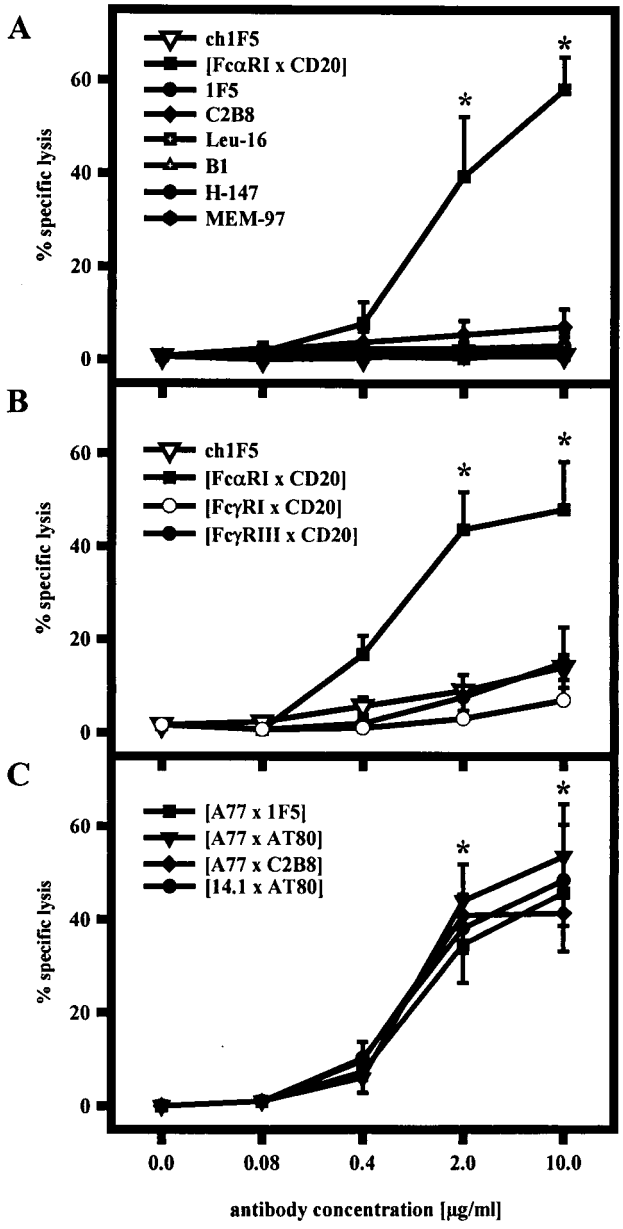

FIGURE 2. (FcαRI × CD20) BsAb recruited PMN as effector cells for CD20- directed therapy. Lysis of the mature B cell line ARH-77 in the presence of an (A77 × 1F5) BsAb against FcαRI and CD20 was compared with killing by murine 1F5 (mIgG2a), Leu-16 (mIgG1), B1 (mIgG2a), H-147 (mIgG3), MEM-97 (mIgG1), by mouse/human chimeric Abs 1F5 or C2B8 (both hIgG1) (*A*), or by FcγRI-, or FcγRIII-directed BsAbs (*B*), all targeting CD20. Additional (FcαRI × CD20) BsAbs were analyzed using A77 or 14.1 (both CD89), and AT80 or C2B8 (both CD20) (*C*). Isolated PMN from healthy donors, either unstimulated (*A*) or in the presence of 50 U/ml GM-CSF (*C*), or from G-CSF-treated patients (*B*) were used at an E:T ratio of 40:1. Significant lysis, indicated by ∗, was observed with the (FcαRI × CD20) BsAbs, but not with any of the other Ab constructs. Each part of the figure represents results as mean ± SEM of experiments with at least three different donors.

Comparison of CD20 mAbs and FcγR-directed BsAbs with (FcαRI × CD20) BsAb

In previous experiments, we found neutrophils to mediate ADCC with mouse/human chimeric IgG1 Abs, but especially G-CSF-primed neutrophils were less effective with this isotype than, e.g., with murine IgG2a or with FcγRI-directed BsAbs (18). As recent results in solid tumor models showed the most potent triggering of PMN-mediated cytotoxicity with FcαRI-directed BsAbs (21, 31), we tested whether (FcαRI × CD20) BsAb could overcome neutrophils' target Ag restriction in killing malignant B cells. As demonstrated in Fig. 2*A*, isolated PMNs from healthy donors proved to be potent cytotoxic effector cells against ARH-77 cells with (FcαRI × CD20) BsAb, whereas the parental 1F5 (mIgG2a) Ab, the respective chimeric human IgG1 construct, other murine CD20 Abs of different isotypes as well as the clinically effective mouse/human chimeric Ab C2B8 (hIgG1) were unable to recruit PMNs as cytotoxic effector cells. This raised the question of whether FcγRI- or FcγRIII-directed BsAbs could induce lysis of malignant B cells via the CD20 target Ag using activated FcγRI-expressing PMNs from G-CSF-primed patients. As expected, these effector cells induced high levels of target cell killing with (FcγRI × HLA class II) BsAb (98 ± 2% specific lysis at 2 μg/ml, $n = 4$). However, against the CD20 target Ag, only the FcαRI-directed BsAb proved effective (Fig. 2*B*). To exclude that this cytotoxic activity was a particular feature of the (A77 × 1F5) bispecific construct, other (FcαRI × CD20) derivatives were generated, including a novel fully human CD89 Ab. As displayed in Fig. 2*C*, all four bispecific constructs were similarly effective in mediating killing of malignant B cells.

A broad range of malignant B cells is susceptible for (FcαRI × CD20) BsAb-mediated cytotoxicity

Lysis of the B cell lines BALL, BJAB, RAJI (both Burkitt's lymphoma), CESS, RM-1, and ARH-77 (all three mature B cell lines) was compared using isolated PMNs from GM-CSF-treated patients in the presence of murine Ab 1F5 (mIgG2a), mouse/human chimeric 1F5 (hIgG1), or the respective (FcαRI × CD20) BsAb (all at 2 μg/ml). All these B cell lines strongly expressed the CD20 Ag (RFI ranging from 14.3 for ARH-77 to 41.4 for RAJI). With the FcαRI-directed BsAb, PMNs lysed all these B cell lines (specific lysis from 9 ± 4% for BALL to 92 ± 5% for RAJI), whereas no killing was obtained with the parental or the chimeric 1F5 Ab (Fig. 3). FcαRI-mediated lysis of different B cell lines did not correlate to their CD20 expression level.

Myeloid growth factors stimulate cytotoxicity via (FcαRI × CD20) BsAb

As neutrophil numbers and their functional capacity can be enhanced by application of the myeloid growth factors G-CSF or GM-CSF, lysis with (FcαRI × CD20) BsAb was investigated with whole blood from cytokine-treated patients or from healthy donors

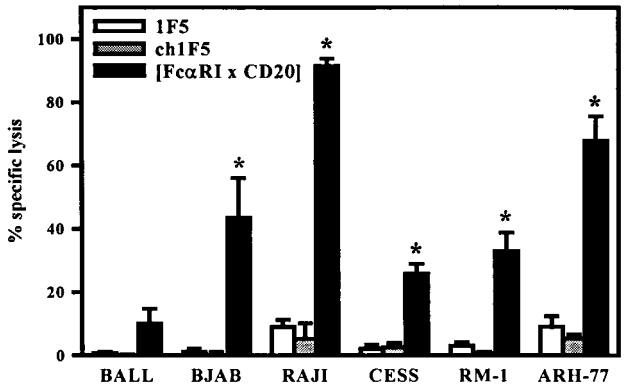

FIGURE 3. A broad range of malignant B cell lines was susceptible to (FcαRI × CD20) BsAb-mediated cytotoxicity. Lysis of CD20-positive B cell lines BALL, BJAB, RAJI (both Burkitt's lymphoma), CESS, RM-1 or ARH-77 (all three mature B cells) was compared using isolated PMN from GM-CSF-treated patients in the presence of murine (1F5) or chimeric (ch1F5) CD20 Ab 1F5, or the respective FcαRI-directed BsAb (FcαRI × CD20) (all at 2 μg/ml). In the presence of the FcαRI-directed BsAb, PMN-mediated lysis against all tested B cell lines (significance indicated by ∗). However, PMN were not effective with the parental or the chimeric CD20 Ab. Data are presented as mean ± SEM of three experiments with different donors.

as effector source. As expected, total leukocyte and PMN counts were significantly higher in growth factor-treated patients compared with those in healthy donors (19,900 ± 3,600/μl; 21,100 ± 2,400/μl; 6,700 ± 600 and 16,700 ± 3,200/μl; 13,800 ± 2,300/μl; 4,300 ± 600/μl for G-CSF, GM-CSF, and healthy donors, $n = 6$, respectively). Importantly, cytotoxicity in blood from cytokine-treated patients was significantly enhanced compared with healthy donor blood, and occurred at 25-fold lower Ab concentrations (Fig. 4). In these experiments, no plasma-mediated lysis was observed, and cell-mediated cytotoxicity resided predominantly in the numerically expanded PMN fraction (data not shown). Interestingly, when analyzed at constant E:T cell ratios, GM-CSF- but not G-CSF-primed PMNs were significantly more effective with the FcαRI-directed BsAb than healthy donor PMNs (68 ± 8, 34 ± 7, and 32 ± 9%, $n = 8$, respectively). Importantly, cytotoxicity by the chimeric CD20 Ab was not enhanced in blood from growth factor-treated patients compared with healthy individuals (11 ± 8, 5 ± 5, and 5 ± 2% specific lysis in the presence of 2 μg/ml of chimeric 1F5 with whole blood from healthy donors, G-CSF-, or GM-CSF-treated patients, $n = 4$ triplets of donors, respectively).

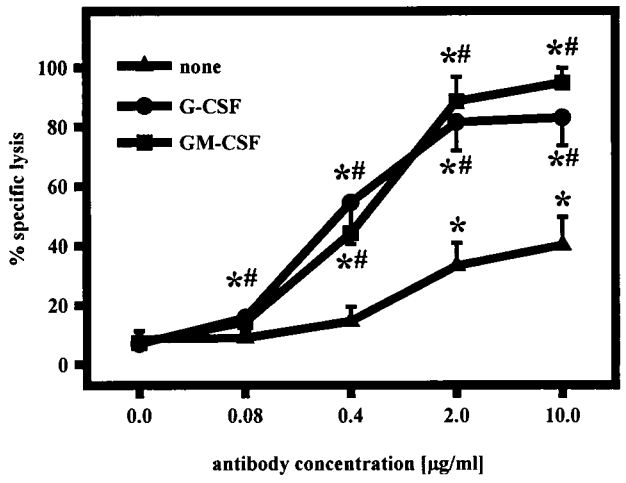

FIGURE 4. The myeloid growth factors G-CSF or GM-CSF stimulate killing of malignant B cells in the presence of (FcαRI × CD20) BsAb. Patients treated with rhG-CSF (G-CSF) or rhGM-CSF (GM-CSF) were compared with healthy donors (none) in their capacity to mediate ADCC against ARH-77 lymphoma cells in the presence of (FcαRI × CD20) BsAb. With blood from cytokine-treated patients, significant lysis (indicated by ∗) occurred at 25-fold lower Ab concentrations and was significantly higher (indicated by #) than with healthy donor blood. Results from experiments with three different triplets of donors are presented as mean ± SEM.

Comparing different B cell-related Ags in ADCC with FcαRI-directed BsAbs

Experiments with mAbs and FcγRI-directed BsAbs showed neutrophils to mediate killing of malignant B cells only with HLA class II-directed Abs (17, 18). Results with the (FcαRI × CD20) BsAb encouraged us to investigate other B cell-related Ags as targets for FcαRI-directed BsAbs. To obtain optimal activation of the effector cell system, whole blood from GM-CSF-treated patients was used for these experiments. Again, HLA class II proved to be the most effective target Ag with high levels of killing occurring at very low Ab concentrations (Fig. 5). However, importantly, significant tumor cell lysis was now also observed with (FcαRI × CD20) and, to a limited extend, with (FcαRI × CD19) BsAbs, whereas the (FcαRI × CD37) BsAb was not effective.

Comparing killing of malignant B cells with FcγRI- or FcαRI-directed BsAbs using blood from mice transgenic for human FcαRI and FcγRI

Syngenic animal models may provide important information for relevant details of clinical trials, provided these models closely reflect the human situation. Blood from G-CSF-treated transgenic mice expressing both human FcαRI and human FcγRI was analyzed as effector source against ARH-77 malignant B cells using FcαRI- or FcγRI-directed BsAbs against CD20. Like neutrophils from G-CSF-treated patients, PMNs from these transgenic animals expressed comparable levels of human FcαRI and human FcγRI (Fig. 6*A*). For control, blood from transgenic animals mediated efficient lysis with (FcγRI × HLA class II) BsAb (32 ± 7%, $n =$

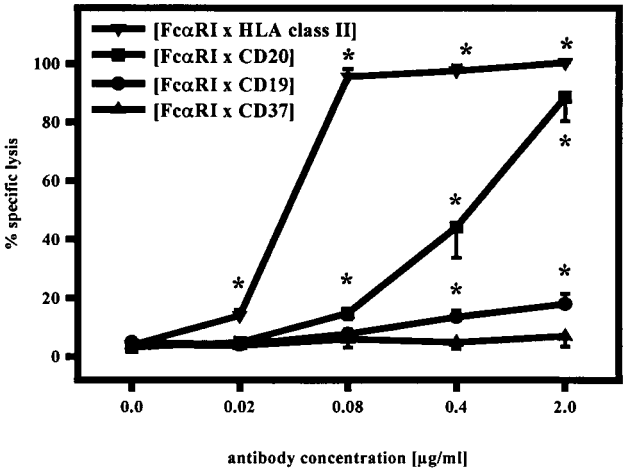

FIGURE 5. Comparing different B cell-related Ags in ADCC with FcαRI-directed BsAb. Lysis of mature B cell line ARH-77 was monitored in 3 h ^{51}Cr release assays using whole blood from three different GM-CSF-treated patients as effector source. FcαRI-directed BsAbs against CD19, CD20, CD37, or MHC class II, respectively, were used at concentrations from 0.02 to 2.0 μg/ml. Results are presented as mean ± SEM; significant lysis is marked by ∗.

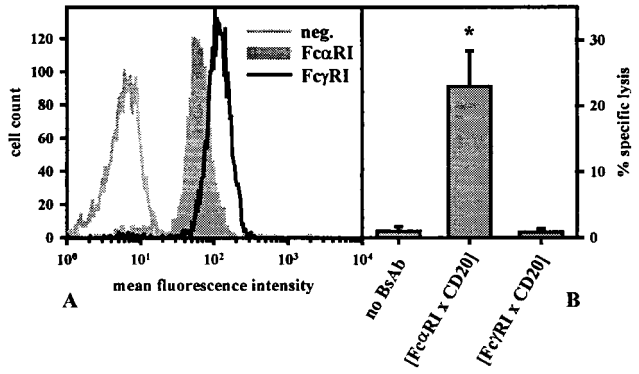

FIGURE 6. Comparing killing with FcγRI- or FcαRI-directed BsAbs with blood from human FcαRI/FcγRI double-transgenic mice. After 4 days of G-CSF priming, expression of human FcαRI or FcγRI on leukocytes from FcαRI/FcγRI double-transgenic mice was compared by indirect immunofluorescence using A77 or 22, respectively. PE-labeled Gr-1 served to identify PMN (*A*). In cytotoxicity assays against ARH-77 B cells using CD20-directed BsAbs (*B*), the FcαRI-, but not the FcγRI-, directed BsAb (both at 2 μg/ml) mediated significant lysis (indicated by $*$). Results are presented as mean $\pm$ SEM of three experiments.

6). However, with CD20 as target Ag, only the FcαRI- but not the FcγRI-directed construct was effective (Fig. 6*B*). Looking for a possible explanation for the superior capacity of the FcαRI transgene to trigger ADCC, the ability of FcαRI and FcγRI to initiate an early signaling event was assessed. Cross-linking FcαRI, expressed on FcαRI/FcγRI double-transgenic PMNs, triggered a very rapid increase in intracellular free calcium levels ($[Ca^{2+}]_i$). Even though cross-linking of FcγRI on the same cells led to increased $[Ca^{2+}]_i$ levels as well, this rise was always delayed compared with FcαRI (Fig. 7), irrespective of the concentration of the cross-linking goat anti-mouse Ab.

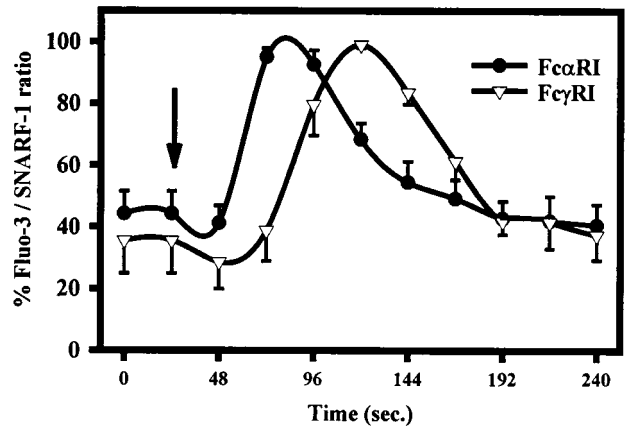

FIGURE 7. Cross-linking of FcαRI leads to rapid calcium mobilization. Intracellular calcium levels ($[Ca^{2+}]_i$) were measured after cross-linking FcαRI (●) or FcγRI (▽). Murine white blood cells were labeled with Fluo-3 and SNARF-1. After incubation with A77 or 22 to stain FcαRI or FcγRI, respectively, cells were resuspended in calcium mobilization buffer. PMN were identified by forward and side scatter profiles, baseline values were established, and after 24 s the cross-linking goat anti-mouse IgG1 Ab was added (arrow) at 4 μg/ml. This consistently led to a rapid rise in $[Ca^{2+}]_i$ after cross-linking FcαRI, and a delayed $[Ca^{2+}]_i$ increase after cross-linking FcγRI. Results from three separate experiments are shown as mean $\pm$ SEM of "% Fluo-3/SNARF-1 ratio," calculated as described in *Materials and Methods*.

Discussion

Previously we reported that neutrophils effectively killed malignant B cells with Abs against HLA class II or against related molecules such as 1D10, Lym-1, or Lym-2, but that they were completely ineffective with IgG Abs or FcγRI-directed BsAbs against "classical" B cell Ags, including CD20 (17, 18). Here we show that this Ag restriction in neutrophil-mediated lysis can be overcome by targeting FcαRI (CD89) instead of FcγRI (CD64). The high cytotoxic activity of (FcαRI $\times$ CD20) BsAbs was confirmed using four different derivatives, including the clinically effective CD20 Ab (C2B8) and a novel fully human CD89 Ab (14.1). Together, there is a clear hierarchy in lysis via different target molecules on tumor cells (with HLA class II $>$ CD20 $>$ CD19 $>$ CD37), as well as in cytotoxic trigger molecules on effector cells (with FcαRI $>$ FcγRI), the latter more obvious with suboptimal target Ags. Further complexity is introduced by different effects of G-CSF and GM-CSF on FcR expression and function. Thus, G-CSF but not GM-CSF induced expression of FcγRI and enhanced lysis via FcγRI-directed BsAbs. In contrast, FcαRI-mediated killing was increased by both G-CSF and GM-CSF, although FcαRI expression was not increased by either cytokine. Increased killing was caused by higher effector cell numbers and enhanced cytotoxicity per cell in the case of GM-CSF, but was only due to increased numbers of effector cells (which were not stimulated compared with healthy donor cells) in the case of G-CSF.

Similar results as with human effector cells were obtained when BsAb-mediated killing of B cells was analyzed with blood from double-transgenic mice expressing both human FcγRI and human FcαRI, indicating that these differences in the killing capacity were truly trigger molecule dependent. Both FcαRI (CD89) and FcγRI (CD64) belong to the family of multi-chain immune recognition receptors (MIRR), in which ligand-specific α-chains form receptor complexes with shared immunoreceptor tyrosine-based activation motif-containing molecules named β-, γ-, or ζ-chains in the case of FcRs. These signaling molecules, of which the FcR γ-chain is most widely expressed, couple these membrane receptors to the intracellular signaling machinery of Src and Syk protein tyrosine kinases (32). Interestingly, FcR γ-chain facilitates surface expression and signaling of both FcαRI and FcγRI (33, 34), suggesting that both receptors activate similar immunoreceptor tyrosine-based activation motif-dependent intracellular signaling pathways (35). Reasons for the observed differences between both FcRs in the successful generation of a cytolytic cascade are not defined at the moment and were most striking for G-CSF-primed PMNs, which expressed comparable levels of both FcγRI and FcαRI. Potential explanations include 1) different killing mechanisms of PMNs activated by FcαRI- compared with FcγRI-directed BsAbs; 2) improved PMN activation via FcαRI either by recruitment of additional signaling pathways or by better interaction between FcαRI and the common FcR γ-chain (33); and 3) different on- and off-rates of the FcαRI- relative to the FcγRI-directed Ab, leading to qualitative differences in FcR triggering (as has been documented for T cell receptor-mediated cell activation; Ref. 36). Our observation that FcαRI cross-linking consistently leads to more rapid rises in $[Ca^{2+}]_i$ supports the idea that FcαRI triggers signaling pathways more efficiently than FcγRI. Interestingly, the difference in the time to maximal $[Ca^{2+}]_i$ levels was more pronounced with lower concentrations of cross-linking Ab, suggesting that FcαRI may be a more potent trigger molecule in situations of limited opsonization with BsAb. Moreover, FcαRI was also found to be

more effective in the generation of an oxidative burst than Fcγ receptors (37, 38). However, reactive oxygen products appeared not involved in neutrophil-mediated lysis because PMNs from patients with chronic granulomatous disease were not impaired in killing malignant B cells (our unpublished observations). Generation of novel reagents including FcγRI- or FcαRI-directed Abs with different affinities, and chimeric Abs of human IgG and IgA isotypes (as natural ligands for these receptors), may help to address some of these questions.

At the moment, chimeric CD20 Abs are probably the best example that unconjugated mAbs can constitute an additional treatment option in oncology. However, clinical results vary and seem to depend, e.g., on patients' histological subtype, indicating that further improvements of efficacy are needed (30, 39). In vivo, therapeutic Abs compete with high levels of endogenous Igs for binding to FcγR. Furthermore, Fcγ receptors are also expressed on cells lacking cytotoxic activity such as platelets or B cells, and some Fcγ receptor isoforms expressed on cytotoxic cells (e.g., FcγRIIb, FcγRIIIb) bind Igs, but do not trigger cytotoxicity, both potentially scavenging therapeutic Ab. This manuscript describes that an (FcαRI × CD20) BsAb is more effective than the respective mouse/human chimeric IgG1 construct in directly killing a broad range of B cell lines, especially when target cells were more complement resistant. Interestingly, PMN-mediated killing of B cells does not appear to be a simple cross-linking phenomenon of target Ags, as indicated by the lack of activity of the (FcγRIII × CD20) and (FcγRI × CD20) derivatives. In addition, FcαRI-mediated lysis was significantly enhanced in blood from G-CSF- or GM-CSF-treated patients. The reason for this enhanced killing during growth factor therapy lies in the fact that FcαRI-directed BsAbs, but not chimeric CD20 Abs, recruited neutrophils as major cytotoxic cell population. Generation of human FcγRI (23), human FcαRI (24), and human FcαRI/FcγRI double-transgenic mice (this manuscript) will help to establish relevant syngenic animal models for the evaluation of BsAb approaches, as ex vivo results with blood from these animals closely reflected the human situation. These animal models are expected to provide relevant information on important details for future clinical trials such as dosing of BsAbs and timing of Ab and cytokine applications. A clinical phase I trial with an (FcαRI × CD20) BsAb in combination with GM-CSF is expected to commence soon. Provided that these phase I data show an acceptable toxicity profile as found in similar studies with FcγRI-directed BsAbs in solid-tumor patients (40, 41), this combination may become a promising approach to enhance efficacy of CD20-directed lymphoma therapy.

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