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ORIGINAL ARTICLE

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Lysis of murine B lymphoma cells by transgenic phagocytes via a human FcγRI×murine MHC class II bispecific antibody

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Abstract The class I IgG receptor (FcγRI) on cytotoxic effector cells has been reported to initiate destruction of tumour cells by effector cells in vitro. We are aiming at developing an immunocompetent model to evaluate the cytotoxic capacity of human FcγRI for the rejection of tumour cells in vivo. Therefore, we recently generated a transgenic mouse strain expressing human FcγRI on monocytes, macrophages, and neutrophils. In these mice, the human receptor is up-regulated by granulocyte-colony-stimulating factor (G-CSF) and is able to trigger cellular responses. Subsequently, in the present study the B cell lymphoma IIA1.6 cell line is selected as a tumour target, and a human FcγRI-directed antitumour bispecific antibody (bsAb) is constructed and characterized. Fab′ fragments of mAb 22, which bind hFcγRI at an epitope that is distinct from the ligand binding site, were chemically linked to Fab′ fragments of rat anti-(mMHC class II antigens) mAb M5/114, yielding bsAb 22×M5/114. This bsAb was able to bind simultaneously to hFcγRI and mMHC class II antigens in a dose-dependent fashion. Binding of 22×M5/114 to FcγRI was not inhibited in the presence of human IgG. It is important to note that, MHC-class-II-expressing IIA1.6 lymphoma cells were lysed by whole blood from G-CSF-treated transgenic mice in the presence of bsAb 22×M5/114. No lysis by whole blood from non-transgenic mice or from transgenic animals that had not received G-CSF was observed. These results indicate that human FcγRI is able to mediate lysis of murine IIA1.6 lymphoma cells by transgenic effector cells via bsAb 22×M5/114. A trial with transgenic mice, evaluating the efficacy of these hFcγRI-directed bsAb in combination with G-CSF for treatment of IIA1.6 B cell lymphoma, is currently in progress.

Key words Fc receptor · CD64 · Transgenic · Bispecific antibody · B cell lymphoma

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Introduction

Bispecific antibodies (bsAb) targeting the class I IgG receptor (FcγRI; CD64) on cytotoxic effector cells to tumour antigens on malignant cells effectively promote lysis of tumour cells in vitro [4]. The safety of such bsAbs in patients with malignancies over-expressing HER-2/neu or epidermal growth factor receptor (EGF-R) is currently being evaluated in phase I/II studies [2, 19]. Preclinical investigations testing the efficacy of bsAb that target human effector cell antigens, have mainly been confined to immunodeficient SCID (severe combined immunodeficient) or nude mice transplanted with human immune cells [17, 21]. However, only limited numbers of human cells repopulate appropriate organs and these mice may, therefore, not represent optimal models [15]. Moreover, immunotherapy based on bsAb and exploiting immune effector mechanisms would best be studied in immunocompetent animals. The approach addressed here, to evaluate the therapeutic potential of FcγRI-expressing cells with bsAb, uses an immunocompetent human FcγRI transgenic mouse strain. In these mice, cellular expression of human (h) FcγRI and its regulation by cytokines closely parallel these processes in humans. The receptor is present on monocytes/macrophages and in low numbers on neutrophils, on which it can be up-regulated by application of G-CSF. It is important to note that the receptor triggers

phagocytosis and antibody-dependent cellular cytotoxicity (ADCC) by transgenic macrophages and neutrophils [11, 12].

As a tumour model we selected the murine B cell lymphoma IIA1.6, a variant of the well-known A20 lymphoma [13]. Malignant B cells are relatively accessible to antibodies and immune effector cells, and are sensitive to lysis by effector mechanisms [9]. Previous studies have demonstrated that malignant B cells are killed by activated neutrophils in the presence of MHC class II antibodies, but not via antibodies to most other B cell antigens, including CD19, CD20 and CD37. This target antigen restriction was not observed with mononuclear effector cells, which lysed malignant B cells with antibodies to all the target antigens tested [5]. To exploit optimally the cytotoxic activity of FcγRI-expressing effectors (including neutrophils) against B lymphoma cells, we constructed a bispecific antibody with specificities for both human FcγRI and murine (m) MHC class II. Here, we describe the binding and cytotoxic characteristics of this bsAb, and demonstrate that murine syngeneic B lymphoma cells are effectively killed by human FcγRI-expressing mouse phagocytes via FcγRI bsAb.

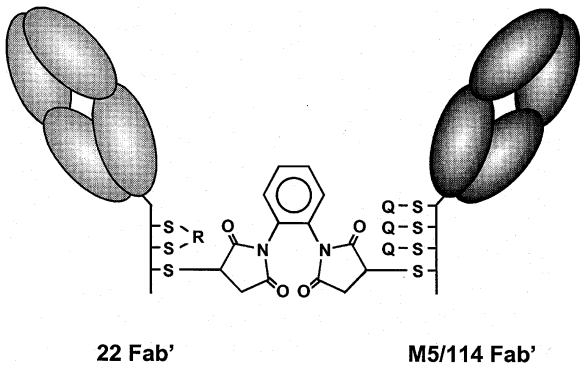

Fig. 1 Schematic representation of bsAb 22×M5/114. Fab′ fragments of mAb 22 and M5/114 were coupled via their hinge-region SH groups using bismaleimide cross-linker. *R* *o*-Phenylenedimaleimide complexed with two hinge SH groups; *Q* alkylated SH groups

Materials and methods

Mice

For the present studies, we used F_1 offspring of crosses between hemizygous human FcγRI transgenic FVB/N (line 52) [11] and BALB/c mice. In these mice, expression of human FcγRI is restricted to monocytes, macrophages and neutrophils. Mice deficient in murine FcγRI and FcγRIII, because of targeted mutations of the FcR γ chain gene (FcR-γ-chain$^-$ mice), were generously provided by Dr. T. Saito (Juntendo University School of Medicine, Chiba, Japan) and have been described elsewhere [18, 20].

Antibodies

Mouse anti-(human FcγRI) mAb 22 (mIgG1; Medarex, Annandale, N.J.) and rat anti-(mouse MHC class II antigens) mAb M5/114 (rIgG2b; ATCC, Rockville, Md.) were used to construct bsAb 22×M5/114. This bsAb was prepared by chemical conjugation of Fab′ fragments of mAb 22 and M5/114 using *o*-phenylenedimaleimide as a bifunctional cross-linker, according to the method described by Glennie et al. [7].

Flow cytometry

To evaluate binding to human FcγRI, U937 cells (ATCC) cultured with interferon γ (250 U/ml for 48 h) were incubated with various concentrations of bsAb 22×M5/114 or mAb 22 on ice. Samples were then stained with fluorescein-isothiocyanate (FITC)-labelled goat $F(ab')_2$ anti-[mouse IgG (H+L)] (Jackson, West Grove, Pa.) and measured on a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.). Binding of 22×M5/114 and M5/114 to mMHC class II was examined using the IIA1.6 murine BALB/c B lymphoma cell line [13] as a target and FITC-labelled mouse $F(ab')_2$ anti-[rat IgG (H+L)] (Jackson) as detecting reagent.

To assess the bispecificity of antibodies, IIA1.6 cells that had been incubated with different concentrations of 22, M5/114, or 22×M5/114, were incubated with soluble recombinant human FcγRI. This soluble receptor was derived from transiently transfected COS cells expressing the extracellular domain of FcγRI fused to C_H2–4 domains of human IgM [8]. After incubation on ice and washing, FITC-labelled CD64 mAb 32.2 (10 μg/ml; Medarex), which binds to an epitope on FcγRI distinct from that recognized by mAb 22, was added. Mean fluorescence intensities were quantified using the flow cytometer.

Antibody-dependent cellular cytotoxicity

Standard short-term ADCC assays were performed as described [12]. Briefly, ^{51}Cr-labelled IIA1.6 lymphoma cells (5×10^3 cells) were mixed with heparin-anticoagulated mouse whole blood (50 μl), and with M5/114 (2 μg/ml) or 22×M5/114 (0.4 μg/ml) in 96-well microtitre plates. After 4 h incubation at 37 °C, cell-free supernatants were harvested to measure ^{51}Cr release and to calculate percentages of specific lysis. In some experiments, mice were treated subcutaneously with rmG-CSF (Amgen, Thousand Oaks, Calif.; 100 μg/kg body weight/day) for 4 days prior to whole-blood isolation [12].

Results and discussion

In order to investigate the therapeutic potential of FcγRI, we (i) generated transgenic BALB/c×FVB/N mice expressing human FcγRI, (ii) developed a tumour model consisting of BALB/c lymphoma IIA1.6 cells, which form splenic tumour nodules, as well as nodules on the liver when implanted intravenously (data not shown), and (iii) prepared a bsAb with specificities for both human FcγRI and murine MHC class II. The bsAb was prepared by joining Fab′ fragments of 22 and M5/114 via stable thioether bonds using *o*-phenylenedimaleimide as cross-linking reagent (Fig. 1). As determined by flow cytometry, bsAb 22×M5/114 bound to hFcγRI-expressing cells and to mMHC-class-II-positive lymphoma cells in a dose-dependent manner (Fig. 2). The detected binding activity of 22×M5/114 to both antigens was predictably less than that of the respective parental mAb. This is because mAb 22 and M5/114 are bivalent, whereas 22×M5/114 is monovalent for each of the two antigens it recognizes. Moreover, the polyclonal reagents used for detection recognize more determinants on a whole IgG (the mAbs) than on a Fab′ fragment (the bsAb)

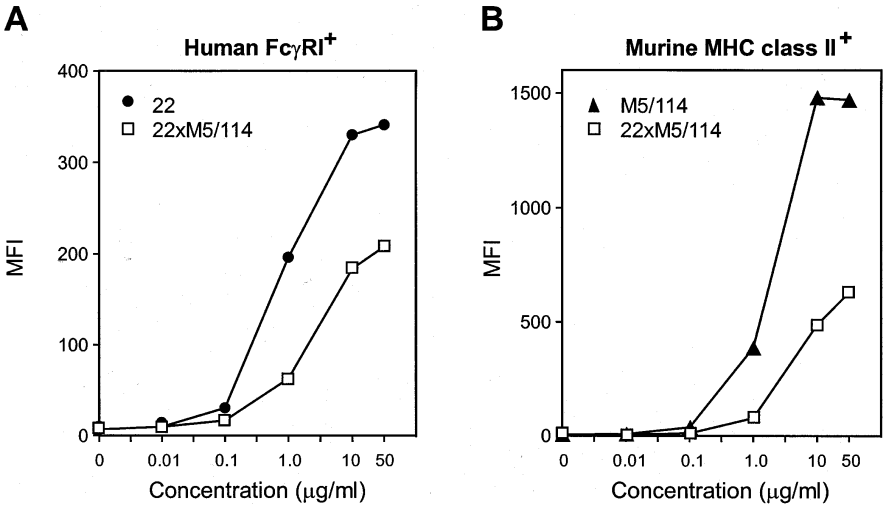

Fig. 2A, B Binding of bsAb 22×M5/114 to cells expressing human FcγRI and murine MHC class II. bsAb 22×M5/114 and mAb 22 bound to human FcγRI on interferon-γ-stimulated U937 cells (**A**) were detected with fluorescein-isothiocyanate(FITC)-conjugated anti-[mouse IgG (H+L)] and analysed by FACScan. B cell lymphoma IIA1.6 cells were used to evaluate binding of bsAb 22×M5/114 and mAb M5/114 to murine MHC class II, which was revealed using FITC-conjugated anti-[rat IgG (H+L)] (**B**). Data are expressed as mean fluorescence intensity (*MFI*)

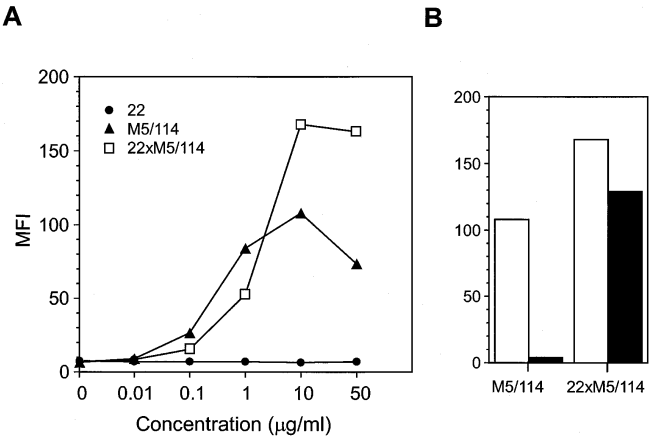

Fig. 3A, B Simultaneous binding of human FcγRI and murine MHC class II by bsAb 22×M5/114 analysed by a sandwich FACScan assay. **A** IIA1.6 cells were incubated with various concentrations of mAb M5/114, 22, or bsAb 22×M5/114, washed, and incubated with soluble recombinant hFcγRI. Binding of hFcγRI to cells was detected by mAb 32.2 conjugated to FITC, which binds FcγRI at an epitope distinct from that recognized by mAb 22 [10]. Cells were analysed by FACScan and data are expressed as mean fluorescence intensity (*MFI*). **B** Binding of soluble hFcγRI to M5/114-coated IIA1.6 cells (10 μg/ml) (*open bars*) was inhibited in the presence of 250 μg/ml human IgG (*closed bars*)

(Fig. 2). To demonstrate that 22×M5/114 binds hFcγRI and mMHC class II simultaneously, the bsAb was tested in a bifunctional assay using the flow cytometer. Lymphoma B cells binding different amounts of 22×M5/114 were allowed to react with soluble hFcγRI. Figure 3A shows that the bound human receptor could be detected in this dual-binding assay, indicating the bispecific nature of 22×M5/114. Similarly, the parental mAb, M5/114 (rat IgG2b), also binds both to MHC class II and to FcγRI (Fig. 3A). This mAb binds to FcγRI via its Fc portion, which was confirmed by incubating the M5/114-coated cells with soluble hFcγRI in the presence of competing human IgG. In contrast to bsAb 22×M5/114, which recognizes FcγRI at a site outside the ligand-binding place [10], mAb M5/114 was no longer able to bind soluble FcγRI under these conditions (Fig. 3B).

The ability of hFcγRI to trigger cytotoxicity of transgenic effectors against murine B lymphoma cells was examined in a whole-blood ADCC assay. This assay represents a close approximation to evaluating ADCC events in a lymphoma situation in vivo. Figure 4 illustrates that whole blood isolated from G-CSF-treated transgenic mice lysed IIA1.6 lymphoma cells in the presence of bsAb 22×M5/114. Tumor cells were not lysed by whole blood from G-CSF-treated non-transgenic mice, nor by whole blood from mice that had not received G-CSF (Fig. 4). Since G-CSF increases neutrophil numbers and up-regulates hFcγRI [14], these data suggest that IIA1.6 lymphoma cells are killed by hFcγRI-expressing neutrophils. As a control, IIA1.6 cells were killed when incubated with whole blood from transgenic and non-transgenic mice in the presence of mAb M5/114. However, only blood isolated from mice that had been treated with G-CSF induced significant levels of lysis (Fig. 4). Increased neutrophil numbers, resulting in increased effector-to-target ratios, probably underlie this effect. Reduced killing of IIA1.6 lymphoma cells via mAb M5/114 was observed when whole blood from FcR-γ-chain-deficient mice (lacking murine FcγRI and FcγRIII [18]) was used, indicating that these receptors are involved in mAb M5/114-mediated ADCC (Fig. 4). We are currently examining the mechanisms of FcγRI-mediated lymphoma killing by isolated transgenic effector cells. For example, activated macrophages from hFcγRI transgenic mice are able to inhibit proliferation of IIA1.6 lymphoma cells in vitro when cultured with bsAb 22×M5/114. When laser-scanning confocal imaging was used ingested IIA1.6 cells were readily detectable inside these macrophages (data not shown).

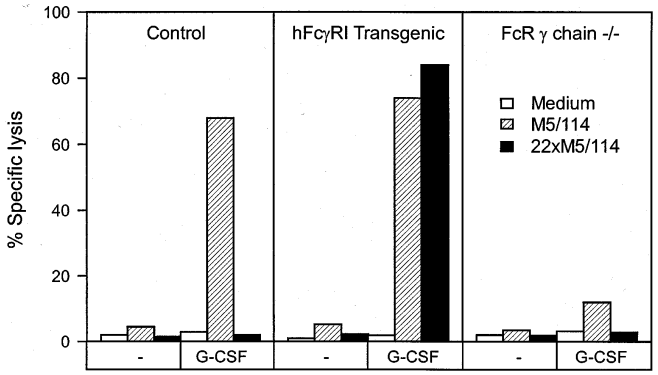

Fig. 4 Whole blood antibody-dependent cellular cytotoxicity of IIA1.6 lymphoma cells. Heparin-anticoagulated whole blood (50 µl) from non-transgenic control, hFcγRI transgenic, or from FcR-γ-chain-deficient mice was incubated with ^{51}Cr-labelled IIA1.6 target cells in the presence of bsAb 22×M5/114 (0.4 µg/ml) or mAb M5/114 (2.5 µg/ml). After 4 h, percentage specific lysis was determined. *G-CSF* results obtained with blood isolated from granulocyte-colony-stimulating-factor-treated mice (100 µg/kg^{-1}/day^{-1}; for 4 days). □ medium; ▨ 115/114; ■ 22×115/114

Previous reports have demonstrated therapeutic effects of MHC class II antibodies in xenotransplanted [6] and syngeneic [1] B cell lymphoma models. Despite the constitutive expression of MHC class II antigens on "professional" antigen-presenting cells, no relevant toxicity or immunocompromising effects of MHC class II antibodies have been observed in these animal models [1]. In addition, studies in human patients with unconjugated and radioactively-labelled HLA class II antibodies showed no other toxicity than that related to irradiation [3]. Serious toxic side-effects, however, were observed in non-human primates treated with HLA-DR antibodies, but these effects were related to complement activation on endothelial cells [16]. Since Fab′ antibody fragments generally do not activate complement, the presented bispecific antibody composed of such fragments may be a promising candidate for treatment of B cell lymphoma. We have recently demonstrated that human FcγRI-expressing effector cells can be targeted by in vivo in transgenic mice [12]. By combined administration of anti-FcγRI bispecific antibodies and G-CSF, tumour antigen-specific neutrophils were generated that readily killed human tumour cells in vitro. These results, together with the data presented here, led us to investigate FcγRI-directed bispecific antibody therapy of B cell lymphoma in human FcγRI transgenic immunocompetent mice. A trial which includes transgenic and non-transgenic control mice, testing the efficacy of bsAb 22×M5/114 administered either alone or in combination with G-CSF, is currently in progress.

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