Characterization of BIS20x3, a bi-specific antibody activating and retargeting T-cells to CD20-positive B-cells

S Withoff1, MNA Bijman1, AJ Stel1, L Delahaye1, A Calogero1, MWA de Jonge2, BJ Kroesen1 and L de Leij1

1University Hospital Groningen, Department of Pathology and Laboratory Medicine, Section Medical Biology – Laboratory Tumor Immunology, Hanzeplein 1, 9713 GZ Groningen, The Netherlands; 2IQ Corporation, Zernikepark 6b, 9747 AN Groningen, The Netherlands

Summary This paper describes a bi-specific antibody, which was called BIS20x3. It retargets CD3-positive cells (T-cells) to CD20-positive cells and was obtained by hybrid–hybridoma fusion. BIS20x3 could be isolated readily from quadroma culture supematant and retained all the signalling characteristics associated with both of its chains. Cross-linking of BIS20x3 on Ramos cells leads to DNA fragmentation percentages similar to those obtained after Rituximab-cross-linking. Cross-linking of BIS20x3 on T-cells using cross-linking F(ab′)2-fragments induced T-cell activation. Indirect cross-linking of T-cell-bound BIS20x3 via Ramos cells hyper-activated the T-cells. Furthermore, it was demonstrated that BIS20x3 effectively re-targets T-cells to B-cells, leading to high B-cell cytotoxicity. The results presented in this paper show that BIS20x3 is fully functional in retargeting T-cells to B-cells and suggest that B-cell lymphomas may represent ideal targets for T-cell retargeting bi-specific antibodies, because the retargeted T-cell is maximally stimulated in the presence of B-cells. Additionally, since B-cells may up-regulate CD95/FasL expression upon binding of CD20-directed antibodies, B-cells will become even more sensitive for T-cell mediated killing via CD95L/FasL, and therefore supports the intention to use T-cell retargeting bi-specific antibodies recognizing CD20 on B-cell malignancies as a treatment modality for these diseases. © 2001 Cancer Research Campaign

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The concept of using antibodies as ‘magic bullets’ for cancer therapy has shown a remarkable revival. Recently highly encouraging results have been reported with antibodies targeting EGFR/EpCAM on carcinomas (Edrecolomab; Riethmüller et al, 1998), HER-2/neu on breast cancers (Trastuzumab; Cragg et al, 1999) and CD20 on lymphomas (Rituximab; Rosen, 1999). Impressive response rates are obtained with these antibodies alone or in combination with additional chemo-therapeutic and/or radio-therapeutic regimens, without showing significant cytotoxicity.

The most promising of these antibodies are those targeting the B-cell differentiation marker CD20. These antibodies have been used for treatment of non-Hodgkin lymphoma (NHL) patients, as more than 90% of all non-Hodgkin lymphomas are positive for CD20. After Rituximab-treatment 50% of patients with advanced, indolent NHL showed partial or complete responses (Rosen, 1999). Because CD20 is expressed on B-cells during a number of stages of B-cell development, but is absent from B-cell progenitor cells and mature plasma cells (Rudin and Thompson, 1998), anti-CD20-antibodies do compromise the patient’s immune system only to a limited extent.

The effectiveness of Rituximab can probably be explained by its supposed dual effector function. First it is known that antibodies retarget the immune system to antibody-coated cells via their Fc-tail, leading ultimately to death of the cell by complement mediated lysis and/or antibody-dependent cellular cytotoxicity. Secondly, it has been shown that binding of antibodies to CD20 can modulate B-cell proliferation and/or antibody-dependent cellular cytotoxicity and that cross-linking of anti-CD20-antibodies (e.g. by Fc-receptor positive cells) on B-cells can lead to apoptosis of the target cell (Shan et al, 1998, 2000). The latter effects are thought to contribute significantly to the efficiency of treatment with this ‘signalling antibody’ (Cragg et al, 1999).

Although the results obtained with Rituximab are very promising it is important to note that anti-CD20-treatment is not curative and that treated patients may relapse ultimately. In order to improve upon the efficiency of anti-CD20-antibody treatment several strategies have been investigated. One approach is the labelling of the antibodies with radioisotopes to induce local irradiation. In another approach toxins are coupled to the antibodies and by this means delivered to tumour cells. Also combination therapy regimens consisting of Rituximab and chemotherapy have been tested in clinical trials (Rosen, 1999). Although these approaches are indeed more successful (higher response rates were achieved) increased toxicity to normal tissues may be expected.

We have focused in the past on the development of bispecific antibodies which are designed to retarget T-cells to tumour (associated) antigens. In the setting of NHL it might be expected that bispecific antibody-mediated retargeting of T-cells to CD20-positive tumour cells will induce apoptosis more efficiently than CD20-cross-linking alone, because T-cells are equipped to optimally induce apoptosis in target cells.

In this paper we describe the production and characterization of the bi-specific antibody BIS20x3 via hybrid hybridoma techniques. BIS20x3 recognizes CD20 with one chain and CD3e
MATERIALS AND METHODS

Culture media and other reagents
RPMI 1640 medium (containing 25 mM Heps and L-glutamin) and 100x HT-supplement were obtained from Bio-Whittaker (Verviers, Belgium) and FCS from Bodinco BV (Alkmaar, The Netherlands). Culturing ‘additives’ consist of 1 mM sodium pyruvate (Bio-Whittaker), 2 mM L-glutamin (Bio-Whittaker), 0.5 mM β-mercaptoethanol, 0.1 mg ml⁻¹ gentamycin sulphate (Bio-Whittaker), and fungizone (Amphotericin-B, Bristol-Myers Squibb, Woerden, The Netherlands). Hygromycin was purchased from Roche Molecular Biochemicals (Almere, The Netherlands), PFHM-II (protein-free hybridoma medium) containing glutamax-I from Gibco BRL-Life Technologies (Breda, The Netherlands), calcein-AM from Molecular Probes (Leiden, The Netherlands, protein-A (PROSEP®-A) from Bioprocessing Ltd (Immunosource, Zoersel-Halle, Belgium), and Triton X-100, propidium iodide (PI) and RNase-A from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands). Lymfoprep was obtained from Nycomed Pharma AS (Oslo, Norway) and serum- and phenolred-free medium (X-VIVO™) from Bio-Whittaker.

Hybridomas, quadroma and cell lines
Hybridoma and quadroma cell lines were cultured in RPMI 1640 medium containing 15% FCS, 15% ESG (conditioned ES-1 culture medium, IQ-Products, Groningen, The Netherlands), additives (see above), and HT-supplement. All other cell lines were cultured in essentially the same medium, however without conditioned ES-1 culture medium and HT supplement. All cells were cultured at 37°C, in a humidified atmosphere containing 5% CO₂. The EBV-immortalized human B-cell line JY was obtained from Dr L. Bakker (Dept. Tumor Immunology, Nijmegen, the Netherlands). CTLL/CD3e is a mouse T-cell line transfected with cDNA encoding human CD3e (Helfrich et al, 1998). Jurkat-AM, which was obtained from Dr P Schrier (University Hospital Leiden, Leiden, The Netherlands), was cultured in the presence of 0.5 mg ml⁻¹ hygromycin in order to select for NFAT-luc (the reporter gene luciferase under control of the NFAT-promoter) positive cells.

Commercial antibodies
FITC-, TRITC- or PE-labelled anti-IgG1- or anti-IgG2b-antibodies were obtained from Becton Dickinson (Erembodegem-Aalst, Belgium) or from IQ-Products (Groningen, the Netherlands). Goat-anti-mouse-F(ab’2)-fragments (GaM-F(ab’2) and goat-anti-human-F(ab’2)-fragments (GaH-F(ab’2)) used for cross-linking of respectively mouse- and human anti-CD20-antibodies were obtained from Jackson Laboratories (West Grove, PA, USA) as well as anti-human IgM-antibody. The anti-CD3-antibody B-B11 was obtained from IQ-Products.

Hybrid hybridoma fusion, subcloning of quadroma cells and characterization of the antibodies produced by the sub-clones
A quadroma cell line was derived after fusion of the hybridoma cell lines B-ly1 (anti-CD20, IgG1; Poppema and Visser, 1987) and CLB-T3/4.2B (anti-CD3e, IgG2b; van Lier et al, 1987a,b). Fusion was performed by standard hybrid hybridoma fusion techniques as described previously (Kroesen et al, 1993). Briefly, a HGPRT-negative anti-CD3e hybridoma cell line was selected by culturing it in 8-azaguanine containing medium. This hybridoma cell line, unable to grow in hypoxantine, aminopterine- and thymidine-HAT containing medium, was made neomycin resistant by retroviral transfection. This HAT sensitive, neomycin-resistant cell line was then fused with the HAT-resistant, neomycin-sensitive hybridoma cell line B-ly1. Standard polyethyleneeglycol (PEG) fusion procedures were used. Quadroma cells were subcloned in 96-well plates and clones producing both IgG1- and IgG2b-chains were identified by detection of fluorescence after labelling the quadroma cells with FITC- or TRITC-labelled anti-IgG1- or anti-IgG2b-antibodies. One subclone was selected. In order to validate CD20- and CD3e-binding capacity of the antibodies produced by this clone, culture supernatant was harvested and applied to CD20-positive JY cells or CD3e-positive CTLL/CD3e cells.

Partial purification of BIS20x3
Twice weekly antibody-producing cells were seeded in PFHM-II medium. Supernatant was harvested when the number of dead cells exceeded 30%. Approximately 500 ml supernatant was collected and concentrated 10x using the ProVario-3FP concentrator (Filtron Technology BV, Terheyden, the Netherlands). Antibody purification was performed by FPLC (Pharmacia, Uppsala, Sweden). Concentrated supernatant was diluted two-fold in 0.1 M K-phosphate buffer pH 8.2 (equilibration buffer) and loaded onto a protein-A column. Antibodies were eluted using a 0.1 M Na-citrate buffer pH-gradient, decreasing from pH 6 to pH 2. Supernatants of the parental hybridomas were subjected to the same procedure in order to determine at which pH the parental antibodies elute from the column. During elution protein peaks were detected spectrophotometrically (OD280) and 0.5 ml fractions were collected. In each fraction the pH was measured and neutralized by adding increasing volumes of a 1 M K-phosphate-solution (pH 9.4). Each fraction was analysed for the presence of IgG1- and/or IgG2b-isotype antibody chains by FACS analysis (Epicis Elite, Coulter, Hialeah, USA) using the antibodies described above. The presence of the various antibody species was confirmed by SDS/PAGE gel electrophoresis and subsequent Western blotting.

Detection of DNA fragmentation after cross-linking of anti-CD20-antibodies on CD20-positive cells
To induce apoptosis Ramos cells were harvested and incubated for 30 min with the different anti-CD20-antibody concentrations (see Results) at 37°C. The cells were washed to remove unbound antibody and plated at a concentration of 0.5–1 × 10⁵ per well (6 well plates, 2.5 ml per well). Cross-linking was achieved by adding
cross-linking F(ab')2-fragments. The next day the Nicoletti assay was performed to determine the percentage (%) apoptotic cells in the population. This assay is based on detection of a hypoploid DNA peak by FACS analysis (Nicoletti et al, 1991). After 16 h apoptosis induction the cells were washed in PBS containing 1% BSA and resuspended in 0.2% Na-citrate/0.2% Triton X-100/0.2 mg ml⁻¹ RNase-A. DNA was stained by adding 100 μg ml⁻¹ PI. PI fluorescence was measured by FACS.

Detection of T-cell activation after CD3e-cross-linking

A TCR-beta chain negative Jurkat cell line harbouring an NFAT-luciferase reporter gene named Jurkat-AM, was retrovirally transduced with genes encoding the alpha and beta TCR chains from an HLA-A2-restricted anti-MAGE-3 T-cell clone (van der Bruggen et al, 1994). The resulting subline is called Jurkat-AM/T. Transduction restored CD3e-expression on the surface of Jurkat-AM/T as was determined by FACS (not shown). After binding of anti-CD3e-antibodies to Jurkat-AM/T, the NFAT-promoter becomes activated which results in luciferase expression. Approximately 1.5 x 10⁶ cells per well (24 well plates) were activated during the period chosen. When necessary 0.5 x 10⁶ Ramos cells were added. The cells were harvested and washed once. Pellets were frozen at –20°C for at least 15 min to facilitate lysis which was performed in lysis buffer supplied by the manufacturer of the Luciferase Assay System (Promega, Leiden, The Netherlands). Luciferase activity was detected using the same kit. Luminescence was detected on the Anthos Lucy Microplate Luminometer and Photometer (LaTech International, Ringmer, UK).

Cytotoxicity assays

We have used the calcein-release assay (Lichtenfels et al, 1994) as an alternative for the ⁵¹Cr-release assay to demonstrate that BIS20x3 re-targets T-cells to CD20-positive cells. PBLs were isolated from heparin-blood using lymphoprep according to protocols described by the manufacturer. T-cells were activated and expanded by incubation for 3 days in RPMI 1640 medium containing 15% normal human poolserum and 5% WT-32 (anti-CD3e-hybridoma) supernatant (Tax et al, 1983), followed by 2 days culturing in RPMI 1640 medium containing 15% normal human poolserum and 100 IU ml⁻¹ IL-2. JY-cells were used as the CD20-positive target cells. JY cells were isolated, washed and resuspended in X-VIVO-medium at a concentration of 2 x 10⁶ cells ml⁻¹. Calcein-AM was added to the cells in a concentration of 8 μM for 40 min, after which extracellular calcein-AM was removed by washing. Calcein-AM is the acetoxymethylester of calcein which diffuses passively across cell membranes. In the cytosol calcein-AM is converted into the fluorochrome calcein by intracellular esterases. Calcein is retained in cells with intact membranes due to its polar nature. We applied 2 x 10⁴ labelled JY target cells per well to 96-well plates, and varied the number of T-cells (also washed and resuspended in X-VIVO-medium) to obtain varying effector-target cell ratios. After 2 h incubation in the absence or presence of different antibodies, the cells in the plates were pelleted and the supernatant was transferred into a new 96-well plate. Calcein fluorescence in the supernatant was determined on the Bio-Tek FL500 fluorescence plate reader (BioTek Instruments Inc, Burlington, USA; excitation at 485 nm, emission at 530 nm). The % cytototoxicity was calculated using the equation: (F(sample) – F(spontaneous release)) / (F(total lysis) – F(spontaneous release)) x 100% = % cytotoxicity. Total lysis values were obtained by addition of 0.5% Triton X-100 to labelled JY cells. The ⁵¹Cr-release assay was performed as described previously (Kroesen et al, 1995).

RESULTS

Hybrid hybridoma fusion

The quadroma cell line producing BIS20x3 was made by fusion of B-ly1 and CLB-T3/4.2B. The quadroma was screened for expression of both parental antibody chains and cultured after subcloning twice. A clone displaying more than 95% double positive cells was selected as the ‘producer’ cell line. Additionally, culture supernatant was applied to CD20- or CD3e-positive cell lines (JY or CTLL/CD3e respectively). The co-presence of antibody-chain subclasses which do not bind by themselves on the target cells (anti-CD20-chains (of IgG1-subclass) on CTLL/CD3e cells or anti-CD3e-chains (of IgG2b subclass) on JY-cells) suggested that the bi-specific antibody was formed and confirmed the antigen-binding capacity of both chains (results not shown).

Isolation and partial purification of the bispecific antibody (BIS20x3)

Culture supernatants from B-ly1-(anti-CD20), CLB-T3/4.2B- (anti-CD3e) or the quadroma culture were isolated and concentrated. First, samples of the concentrated parental hybridoma supernatants were mixed and loaded onto a protein-A FPLC column to elucidate the pH-elution profile of both parental antibodies. Using FACS and Western blot analysis it was shown that the parental anti-CD20-antibody B-ly1 leaves the column between pH 5.5 and pH 4 and that the anti-CD3e-antibody elutes between pH 3 and 2 (peak at pH 2.5). In Figure 1 the elution profiles are depicted. The results suggest that the parental antibodies can be separated from each other. After loading quadroma supernatant and subsequent antibody elution using the same pH gradient, it was demonstrated that the bi-specific antibody leaves the column at a pH value lower than pH 3.8 indicating that the bi-specific antibody, which was called BIS20x3, could be isolated without ‘contaminating’ B-ly1. However, the BIS20x3 peak overlaps partially with the anti-CD3e-peak. Therefore it was decided to use only the fractions isolated from the first half of the BIS20x3-peak for the experiments described below (more than 90% pure regarding BIS20x3-content). To check the

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functionality of the antigen-binding capacity of both arms of the bispecific antibody, assays were subsequently performed which detect the intracellular signals which are relayed after binding of antibodies to CD20 on B-cells or CD3ε on T-cells, respectively.

Detection of DNA hypo-ploidy after cross-linking of anti-CD20-antibodies on Ramos-cells

It was reported that binding of anti-CD20-antibodies to CD20 and subsequent cross-linking of these antibodies leads to apoptosis induction in Ramos cells (Shan et al, 1998). From Figure 2A and 2B it can be concluded that B-ly1 cross-linking leads to similar DNA-fragmentation percentages as when Rituximab is cross-linked. A dosage of only 0.1–0.5 μg ml⁻¹ B-ly1 or Rituximab is enough to detect apoptotic cells after cross-linking during 16 h. Also shown is that addition of anti-CD20 antibody alone or cross-linking antibody alone does not affect the Ramos cells. As a control anti-IgM-antibody was applied, resulting in high percentages of DNA fragmentation. Also cross-linking of BIS20x3 leads to DNA fragmentation in Ramos cells (Figure 2C), becoming evident at a BIS20x3 concentration of 0.5 μg ml⁻¹. The levels of DNA fragmentation appear to be higher than achieved in Figure 2A, B, however it has to be remarked that the untreated control shows already a relatively high apoptotic fraction in this experiment. The results presented here demonstrate that the anti-CD20 chain of BIS20x3 is functionally active in inducing apoptosis upon cross-linking.

T-cell signalling induced by binding of BIS20x3 to Jurkat-AM/T cells

To demonstrate the functional capacity of the CD3ε-chain of CD20x3 to activate T-cells, a CD3ε-signalling assay was used which was described in the Materials and methods section. Figure 3A shows results obtained after binding of BIS20x3 to CD3ε expressed on the surface of Jurkat-AM/T cells, and subsequent cross-linking of BIS20x3 by goat-anti-mouse-F(ab’²)-fragments. Cross-linking of increasing amounts of BIS20x3 results in increasing levels of T-cell stimulation. As a positive control T-cells were stimulated with WT-32 culture supernatant and as a negative control no antibody was added. Because we also expected indirect CD3ε-cross-linking to occur (and therefore T-cell activation) when Ramos cells were added to BIS20x3-coated Jurkat-AM/T cells, we performed the experiment summarized in Figure 3B. In this graph it is clearly shown that the T-cells are much more efficiently stimulated when BIS20x3 is cross-linked via Ramos cells than when the bi-specific antibody is cross-linked with goat-anti-mouse-F(ab’²)-fragments (Figure 3A), what may be caused by a co-stimulatory capacity of the Ramos cells. The results presented in Figure 3 demonstrate the functional binding of BIS20x3 to CD3ε.

BIS20x3 re-targets PBL derived T-cells to CD20-positive JY cells

In Figure 4 representative results obtained with the calcein release assay are presented. In Figure 4A it is shown that when using the JY cell line, spontaneous release values were observed of approximately 25%. Neither the medium taken from unlabelled JY cell cultures nor medium from unlabelled T-cell cultures displayed

![Figure 2]({{https://example.com/figure2.png}})

**Figure 2** Apoptosis induction in Ramos cells after cross-linking of anti-CD20 antibodies on the cell surface. In the experiment described in Figure 2A apoptosis was induced by cross-linking of B-ly1 which is the parental antibody used for derivation of BIS20x3. In Figure 2B results obtained after cross-linking of Rituximab are described. Figure 2C depicts results obtained after cross-linking of BIS20x3. Abbreviations used: no Abs = no antibodies, Bly = B-ly1, GaM = goat-anti-mouse-F(ab’²)-fragments, IgM = anti-IgM-antibody, Rit = Rituximab, GaH = goat-anti-human-F(ab’²)-fragments, Bis = BIS20x3. The numbers describe the amount of each antibody (or antibody-fragment) applied, in μg ml⁻¹. The data presented in this graph are representative for 3 or more experiments.
background fluorescence. Addition of 1 μg ml⁻¹ B-ly1, CLB-T3/4.2B or a combination of both parental antibodies to wells containing T- and B-cells at an E/T-ratio of 20 had no effect. In Figure 4B a representative CTL-assay is described. It is shown that BIS20x3 re-targets PBL-derived, pre-activated T-cells to JY cells. With increasing effector/target cell ratios increased cytotoxicity %s were observed. These results could be reproduced readily indicating the good reproducibility of this non-radioactive cytotoxicity assay. Additionally, the results obtained by using the calcein release assay were essentially similar to results obtained by ⁵¹Cr-release (not shown). In none of the performed assays Rituximab was able to cause B-cell lysis in the calcein-release assay as it was performed here (essentially the results were similar to those obtained for B-ly1 described in Figure 4A). Finally, Figure 4C shows that the retargeting of T-cells to CD20 positive cells is BIS20x3-concentration-dependent and that cytotoxicity can be detected at BIS20x3 concentrations which are considerably lower (factor 50) than the anti-CD20-antibody concentrations needed to detect DNA fragmentation (Figure 2).

DISCUSSION

The anti-CD20-antibody Rituximab is concerning its ability to induce tumour response rates the most effective anti-cancer antibody
Rituximab treated B-cells more sensitive for antibodies on B-cells, but that the concentration of 5 \text{mg ml}^{-1} than the concentrations used by Shan et al (10 \text{mg ml}^{-1}). In the in vitro setting. However, this may have important consequences for the in vivo setting. Increased \text{Fas} expression could make Rituximab treated B-cells more sensitive for \text{FasL} produced by other cells, especially by T-cells (Shresta et al, 1998). This is an important notion, especially in the context of T-cell re-targeting e.g. using the bi-specific antibody described in this paper.

Although several effector mechanisms seem to be triggered by Rituximab, 50% of NHL-patients do not respond. Recently, also patients with CD20-positive post-transplantation lymphoproliferative disorder (PTLD) were treated with Rituximab and also in this group 30% of the patients did not respond (Milpied et al, 2000). Various escape mechanisms which cause this non-responsiveness can be envisaged (e.g. a relative tumour cell-resistance to apoptosis induction). We hypothesize that the efficacy of treatment will increase when the delivered apoptotic signal can be strengthened. This was expected because in general the percentage of unwanted heavy/light chain combinations is low because each heavy chain up to 10 different immunoglobulin molecules due to shuffling of the heavy and light chains (Kroesen et al, 1998), our purified bi-specific antibody gets too high the binding sites on T- and B-cells escalating clinical studies are planned. If the concentration of bi-specific antibody gets too high the binding sites on T- and B-cells may become saturated without cross-linking the cells to each other.

Although it is theoretically possible that our quadroma produces up to 10 different immunoglobulin molecules due to shuffling of the heavy and light chains (Kroesen et al, 1998), our purified product is very efficient in retargeting T-cells to B-cells (Figure 4). This was expected because in general the percentage of unwanted heavy/light chain combinations is low because each heavy chain has the highest affinity for its own light chain. Moreover, the most important and most abundant ‘contaminants’, the parental mono-specific species, were certainly not isolated (see Figure 1). The experiments above and the high retargeting efficiency therefore suggest that the bispecific antibody is present in abundant amounts. The high cytotoxicity observed may be due to optimal T-cell activation caused by co-stimulatory signals delivered by the B-cells and/or by the increased sensitivity of the B-cells for \text{FasL} after binding of anti-CD20 antibodies. Although we did not address this question in this study it will be interesting to investigate if in the bi-specific setting, the apoptosis-inducing signal induced upon anti-CD20-crosslinking plays an important role or whether the T-cell-mediated signal is far more efficient. Another important question is if BIS20x3 is more efficient in treating lymphomas than CD19\times\text{CD3} bispecific antibodies. Several CD19\times\text{CD3} bispecific antibodies have been described recently (Barbançon et al, 1998; Zech et al, 2000; Löffler et al, 2000) in several antibody-formats (quadroma-derived, bi-specific single-chain and diabody respectively). The findings that CD20 seems to be higher expressed on B-cells compared to CD19 and that CD20 is not internalized after antibody binding in contrast to CD19-antibody complexes are often used as arguments for the use of CD20 in the clinic at present. Its success is most probably due to a diversity of effects which are induced after binding to CD20. First, it has been shown that Rituximab can kill via complement mediated lysis. Very recently the C1q binding site on Rituximab has been identified (Iudsogio et al, 2000). The second mechanism by which Rituximab is thought to elicit target cell death is via antibody-dependent cellular cytotoxicity. Although both mechanisms are potent cell death activating routes it is thought that the efficacy of Rituximab is at least partially due to additional mechanisms which are directly triggered by CD20 upon binding of the antibody. The function of CD20 as a signalling molecule has been investigated by several groups. It was demonstrated that upon binding of anti-CD20 antibodies 95% of all CD20 molecules present on the cell move to lipid rafts in the cell membrane which are known to be important in cellular signalling (Deans et al, 1998). It is thought that CD20 signals indirectly via tyrosine kinases which are known to be present in these rafts and which were shown to interact with CD20 (Deans et al, 1995) or directly by functioning as a Ca^{2+}-channel (Bubien et al, 1993; Tedder and Engel, 1994). Recently it was demonstrated that cross-linking of anti-CD20 antibodies on the surface of B-cells using F(ab')2-fragments or Fc-receptor positive cells induces apoptosis in B-cells (Shan et al, 1998). The same group confirmed that protein tyrosine kinases, increased intracellular Ca^{2+}-concentrations, and caspas are involved (Shan et al, 2000). In the latter paper it was also shown that CD95 (\text{Fas}) becomes up-regulated after cross-linking of anti-CD20-antibodies on B-cells, but that the \text{Fas-FasL} route is not important in the in vitro setting. However, this may have important consequences for the in vivo setting. Increased \text{Fas} expression could make Rituximab treated B-cells more sensitive for \text{FasL} produced by other cells, especially by T-cells (Shresta et al, 1998). This is an important notion, especially in the context of T-cell re-targeting e.g. using the bi-specific antibody described in this paper.
antibodies (e.g. Press et al, 1989). In vivo comparison of CD3xCD19 antibodies with BIS20x3 would be very informative in this matter.

The application of T-cell retargeting anti-CD20 antibodies in therapies for B-cell malignancies may be an improvement upon Rituximab-based therapies. Our finding that T-cells are very efficiently activated in a setting in which B-cells are present is very promising in this respect. This characteristic is probably caused by the fact that unlike most other cancer cells, malignant B-cells may be capable to function as antigen-presenting cells (Chaperot et al, 2000). Also the recent finding that after cross-linking of anti-CD20-antibodies on B-cells Fas is up-regulated in these cells (Shan et al, 2000) could have major implications for T-cell retargeting-based lymphoma therapies, because this will make the B-cells even more sensitive for FasL on activated T-cells.

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REFERENCES

Bubien JK, Zhou LJ, Bell PD, Frizzell RA and Tedder TF (1993) Transfection of the CD20 cell surface molecule in ectopic cell types generates Ca2+ conductance found constitutively in B lymphocytes. J Cell Biol 121: 1121–1132

Chaperot L, Jacob MC, Molens JP, Manches O, Bensa JC and Plumas J (2000) From tumor cell immunogenicity to the generation of antitumor cytotoxic cells in non-Hodgkin’s lymphomas. Leuk Lymphoma 38: 247–263

Cochlovius B, Kipriyanov SM, Stassar MJ, Christ O, Schuhmacher J, Strauss G, Helfrich W, Kroesen BJ, Roovers RC, Westers L, Molema G, Hoevenboom Idusogie EE, Presta LG, Gazzano-Santoro H, Topal K, Wong PY, Ulsch M, Meng YG and Mulkerin MG (2000) Mapping of the C1q binding site on Rituxan, a chimeric antibody with a human IgG1 Fc. J Immunol 164: 4178–4184

Kroesen BJ, Ter Haar A, Spakman H, Willemsen P, Sleijfer DTH, De Vries EGE, Mulder NH, Berendes HH, Limburg PC, the TH and De Leij L (1993) Local antitumour treatment in carcinoma patients with bi-specific-monoclonal-antibody-directed T cells. Cancer Immunol Immunother 37: 400–407

Kroesen BJ, Bakker A, Van Lier RAW, The HT and De Leij L (1995) Bispecific antibody mediated target-cell specific costimulation of resting T-cells via CD5 and CD28. Cancer Res 55: 4409–4415

Kroesen BJ, Helfrich W, Molema G and De Leij L (1998) Bispecific antibodies for treatment of cancer in experimental animal models and man. Adv Drug Deliv Rev 31: 105–129

Lichtenfels R, Biddison WE, Schulz H, Vogt AB and Martin R (1994) CARE-LASS (calcinein-release-assay), an improved fluorescence-based test system to measure cytotoxic T lymphocyte activity. J Immunol Meth 172: 227–239

Löfler A, Kuffer P, Lutterbisse R, Zettl F, Daniel PT, Schwenkenbecher JM, Riethmüller G, Dörken B and Bargou RC (2000) A recombinant bispecific single-chain antibody, CD19xCD3, induces rapid and high lymphoma-directed cytotoxicity by unstimulated T lymphocytes. Blood 95: 2098–2103

Milpied N, Vasseur B, Parquet N, Garnier JL, Antoine C, Quartier P, Carret AS, Boucary D, Faye A, Bourbigot B, Regnere Y, Stoppa AM, Bourguard P, Hurauld de Ligny B, Dubief F, Mathieu-Bone A and LeBlond V (2000) Humanized anti-CD20 monoclonal antibody (Rituximab) in post transplant B-lymphoproliferative disorder: A retrospective analysis on 32 patients. Ann Oncol 11 (suppl): S113–S116

Nicolleti I, Migliorati G, Pagliacci MC, Grignani F and Riccardi C (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Meth 139: 271–279

Poppema S and Visser L (1987) Preparation and application of monoclonal antibodies: B cell panel and parafin tissue reactive panel. Biotest Bull 3: 131–139

Press OW, Farr AG, Borroz KI, Anderson SK and Martin PJ (1989) Endocytosis and degradation of monoclonal antibodies targeting human B-cell malignancies. Cancer Res 49: 4906–4912

Rietmuller G, Holz E, Schlimok G, Schmiegel W, Hofken K, Gruber R, Funke I, Pichlmaier H, Hirche H, Buggisch P, Witte J and Pichlmayr R (1998) Monoclonal antibody therapy for resected Dukes’ C colorectal cancer: seven-year outcome of a multicenter randomized trial. J Clin Oncol 16: 1788–1794

Rosen ST (Guest Ed.) (1999) In: Recent advances and future directions using monoclonal antibodies for B-cell malignancies. Semin Oncol 26 (suppl14): 2–12

Rudin CM and Thompson CD (1998) B-cell development and maturation. Semin Oncol 25: 435–446

Shan D, Ledbetter JA and Press OW (1998) Apoptosis of malignant human B cells by ligation of CD20 with monoclonal antibodies. Blood 91: 1644–1652

Shan D, Ledbetter JA and Press OW (2000) Signaling events involved in anti-CD20-induced apoptosis of malignant human B cells. Cancer Immunol Immunother 48: 673–683

Shresta S, Pham CTN, Thomas DA, Granbreti TA and Ley TJ (1998) How do cytotoxic lymphocytes kill their targets? Curr Opin Immunol 10: 581–587

Tax WJM, Willemse HW, Reekers PPM, Capel PJ and Koene RAP (1983) Polymorphism in mitogenic effect of IgG1 monoclonal antibodies against T3 antigen on human T-cells. Nature 304: 445–447

Tedder TF and Engel P (1994) CD20: A regulator of cell cycle progression of B-lymphocytes. Immunol Today 15: 450–454

Van der Bruggen P, Bastin J, Gajewski T, Coulie P, Boel P, De Smet C, Traversers C, Townsend A and Boon T (1994) A peptide encoded by human gene MAGE-3 and presented by HLA-A2 induces cytolytic T lymphocytes that recognize tumor cells expressing MAGE-3. Eur J Immunol 24: 3038–3043

Van Lier RA, Boot JH, De Groot E and Aarden LA (1987a) Induction of T cell proliferation with anti-CD3 switch-variant monoclonal antibodies: effects of heavy chain isotype in mouse-dependent systems. Eur J Immunol 17: 1599–1604

Van Lier RA, Boot JH, Verhoeven AJ, De Groot ER, Brouwer M and Aarden LA (1987b) Functional studies with anti-CD3 heavy chain isotype switch-variant monoclonal antibodies. Accessory cell-independent induction of interleukin 2 responsiveness in T-cells by epsilon-anti-CD3. J Immunol 139: 2873–2879

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