Electronic Journal of Biotechnology ISSN: 0717-3458
http://www.ejbiotechnology.info
DOI: 10.2225/vol15-issue5-fulltext-3 *RESEARCH ARTICLE*

Complement dependent cytotoxicity activity of therapeutic antibody fragments is acquired by immunogenic glycan coupling

Anthony Courtois[#1,2] · **Stéphanie Gac-Breton**[#1] · **Christian Berthou**[2] · **Jean Guézennec**[1] · **Anne Bordron**[§2] · **Claire Boisset**[§1] ✉

1 IFREMER-Brest Center, Biotechnologies and Marine Molecules laboratory, Technopôle Brest-Iroise, France

2 Brest University Hospital, Cellular Therapy and Immunobiology of Cancer laboratory, Hematological service, France

✉ Corresponding author: Claire.Boisset@ifremer.fr
[#]Present address: Polymaris Biotechnology, Morlaix Chamber of Commerce and Industry, Airport, Morlaix, France.
[§]This authors equally contributed to this work
Received December 29, 2011 / Accepted July 9, 2012
Published online: September 15, 2012
© 2012 by Pontificia Universidad Católica de Valparaíso, Chile

Abstract Oligosaccharides are implicated in the development of the immune response notably in complement activation. Anti-tumoural immunotherapy by monoclonal antibodies (mAbs) offers some advantages to chemotherapy including cell targeting but some of them are inefficient to generate cytotoxicity dependent complement (CDC) known to be important in the antibody's efficacy. The aim of this study is to give a CDC activity of mAb by linkage of a complement activating oligosaccharide to this antibody via a hetero-bifunctional linker allowing control of the conjugation reaction. We worked on non Hodgkin Burkitt's lymphoma as cancer source, Fab fragments of rituximab devoid of complement activity as mAb and the trisaccharide Galα(1→3)Galβ(1→4)GlcNAc as immunogenic glycan. The bioconjugate Fab-Gal was characterized by biochemical methods and we demonstrated that the α-Gal epitope was recognized by seric immunoglobulins. After checking the recognition capacity of the Fab-Gal conjugate for the CD20 epitope, *in vitro* assays were performed to evaluate the activation of the complement cascade by the Fab-Gal conjugate. The effect of this bioconjugate was confirmed by the evaluation of the proliferation response of Burkitt's cell line. The relative facility realization of this strategy represents new approaches to increase activities of mAbs.

Keywords: complement system, Galα(1→3)Gal epitope, immunogenic oligosaccharide, immunotherapy, non-Hodgkin lymphoma, xenoantigen

INTRODUCTION

Complement dependent cytotoxicity (CDC) is very important for the optimal therapeutic monoclonal antibodies (mAb) function (Beum et al. 2008) and is totally conserved even after a chemotherapy treatment. However, this activity is generated by some antibodies (Manches et al. 2003) but not all of them (Cardarelli et al. 2002; Wang et al. 2004). Certain molecules have an immunomodulative efficacy and notably by activating the complement system, among them, some glycans lead to the formation of a membrane attack complex (MAC) (Walpen, et al. 2002; Courtois et al. 2008) as well as to the production of pro-inflammatory molecules (C3a, C4a, C5a). These molecules stimulate various cellular effectors of the immune system such as macrophages, monocytes or natural killer (NK) cells and lead *in fine* to lysis of pathogenic cells (Cholujova et al. 2009).

Certain glycan structures are specifically recognized by natural antibodies and more particularly by immunoglobulin M (IgM) which are potent activators of the classical pathway of the complement system. This activation occurs during xenotransplantation, in pig-to-human transplants, where the transplant is rapidly rejected by the host. This transplant rejection is mainly due to a trisaccharide antigenic structure abundantly expressed at the surface of pig cells: the

Galactoseα(1→3)Galactoseβ(1→4)-*N*-acetylglucosamine (Galα(1→3)Galβ(1→4)GlcNAc) trisaccharide. This trisaccharide is also called a xenoantigen or an α-Gal epitope (Galili, 2001) and natural anti-Gal antibodies are present in human blood. The α-Gal epitope is expressed at a rate of 1 x 10^6 to 30 x 10^6 motifs on the cell surface of most mammalian species, except in humans and Old World primates (Galili et al. 1988). In humans, as much as 1% of total serum IgG is specific to the α-Gal epitope and significant levels of anti-Gal IgM (~1 to 8% of total IgM) and IgA have also been reported (McMorrow et al. 1997). Xenotransplant rejection is mainly due to the anti-Gal IgM (Sandrin et al. 1993) and the immune response depends on the activation of the classical pathway of the complement system which leads to MAC formation and cell lysis.

Published results have shown that tumours modified to express the α-Gal epitope can direct the action of the complement system on tumour cells and be used as a cancer vaccine (Latemple et al. 1996; Galili and Latemple, 1997). Moreover, direction injection of α gal epitope allows destroying some tumours (Abdel-Motal et al. 2009). Burkitt's lymphoma is a very aggressive B-cell non-Hodgkin lymphoma (NHL) requiring intensive and toxic chemotherapy (Sehn, 2008). To improve survival of patients, rituximab, a monoclonal antibody (mAb) directed against CD20 and strongly expressed at the surface of B lymphocytes, has been used alone or in combination with chemical agents with some promising therapeutic effects (Galili et al. 2003; Aldoss et al. 2008). The action of rituximab is currently being examined in other B lymphocyte malignancies and in auto-immune diseases (Edwards et al. 2002; Eisenberg, 2003; Gottardo et al. 2003; Zecca et al. 2003; Zheng et al. 2003). Rituximab mediates cell-killing effects through different mechanisms: apoptosis (Shan et al. 2000) antibody-dependent cellular cytotoxicity (ADCC) (Wang et al. 2008) and CDC (Van Meerten et al. 2006). The components of the serum are even preserved after chemotherapy, what is not the case of cells generating the ADCC; what makes of the CDC an important mechanism of the activity of mAbs.

Various research groups are now working on developing new antibody constructs to amplify antitumor activity, by modifying the protein or oligosaccharide structures of therapeutic antibodies or by vectorizing various molecules, such as immunostimulant proteins (Reiter and Fishelson, 1989; Juhl et al. 1990).

The aim of this work is to demonstrate within a lymphoma model that it makes possible to adduce a CDC activity to any antibody or antibody fragment by covalently coupling a mAb with an immunogenic oligosaccharides via heterobifunctional linkers. Xenoantigens Galα(1→3)Galβ(1→4)GlcNAc were chosen for their high immunogenicity and rituximab for its ability to recognize the antigen CD20 at the cell surface of B lymphocytes. We generated Fab fragments of rituximab because Fab fragments do not possess the Fc part of the rituximab molecule and cannot activate the CDC alone. The resulting conjugate was named Fab-Gal and *in vitro* assays were performed to assess activation of the complement cascade. The effect of this bioconjugate on the proliferation of the Burkitt's lymphoma cell line was also evaluated.

MATERIALS AND METHODS

Generation of Fab fragments

Fab fragments were obtained by immobilized papain digestion (Perbio, Brebieres, France) (Figure 1, step 1), were purified on a protein A column (GE Healthcare Amersham Bioscience, Orsay, France) and dialyzed against phosphate buffer (10 mM, pH 7.4). The fragments were then analyzed under denaturing reducing conditions using 10% SDS-PAGE gel electrophoresis. Protein concentrations were determined using the Bradford colorimetric method (Bradford, 1976).

Functionalization of Fab fragments

Fab fragments were incubated with 46 µL of Traut's reagent (Perbio, Brebieres, France) (2 mg/mL in PBS) for 1 hr at room temperature. Excess Traut's reagent was eliminated by ultra filtration (Figure 1, step 2). Protein concentrations were determined using the Bradford colorimetric method (Bradford, 1976). The number of sulfhydryl groups formed was determined by the measuring the optical density (OD) at 412 nm using Ellman's reagent (Ellman, 1959) (Perbio, Brebieres, France).

N-Deacetylation of the trisaccharide Galα(1→3)Galβ(1→4)GlcNAc

Galα(1→3)Galβ(1→4)GlcNAc (Dextra Laboratories, Reading, UK) (5 mg) was incubated with 1 M NaOH (300 µL) containing 1% of $NaBH_4$ for 1 hr at 80ºC (Figure 1, step 3). The deacetylated trisaccharide was purified by size-exclusion chromatography on a Biogel P2 column (Biorad, Marnes La Coquette, France) and lyophilized. The presence of amine groups was checked with thin layer chromatography on silica plates (Merck, France) was revealed by ninhydrin.

Conjugation of the *N*-deacetylated Galα(1→3)Galβ(1→4)GlcNAc to rituximab Fab fragments

N-deacetylated Galα(1→3)Galβ(1→4)GlcNAc trisaccharide (1 eq.) in PBS was incubated with the heterobifunctional linker, sulfosuccinimidyl 4-[*N*-maleimidomethyl] cyclohexane-1-carboxylate (Sulfo-SMCC) (Perbio, Brebieres, France) (2 eq.) for 2 hrs at room temperature (Figure 1, step 4a). The reaction mixture was then incubated with the functionalized rituximab Fab fragments (0.5 eq.) for 1 hr at room temperature (Figure 1, step 4b).

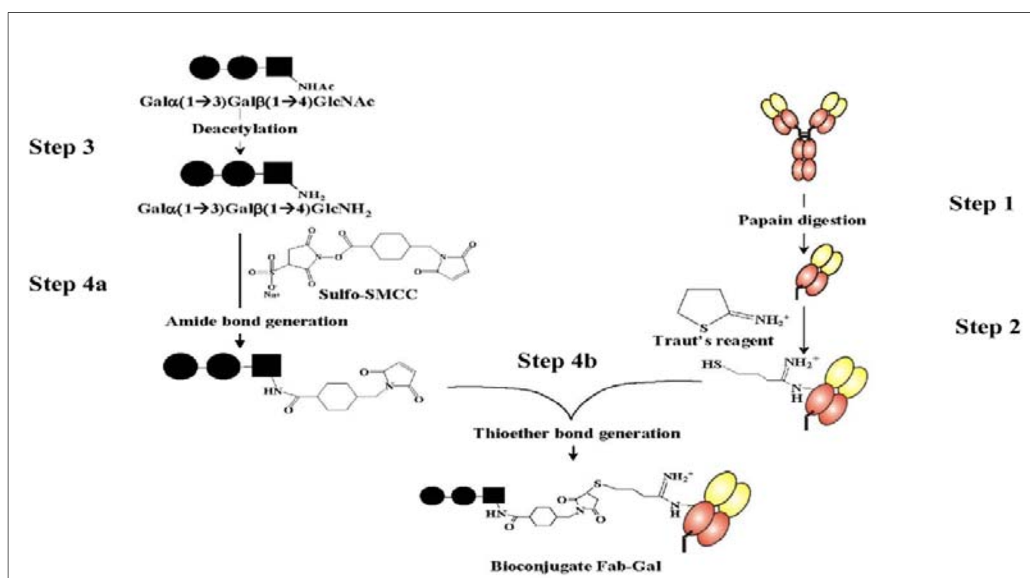

Fig. 1 Conjugation of the xenoantigen to Fab fragments of rituximab. Route of conjugation of one xenoantigen to Fab fragments of rituximab via the heterobifunctional linker sulfosuccinimidyl 4-*N*-maleimidomethyl cyclohexane-1-carboxylate (sulfo-SMCC). Black circles represent galactose and black squares, *N*-acetyl-glucosamine.

Recognition of the α-Gal epitope by immunoglobulins in normal human serum: Ouchterlony immunodiffusion

Briefly, 70 µL of Fab and Fab-Gal samples and 35 µl of Fab-Gal (1 mg/mL in 0.1 M PBS) was loaded in wells on a 1% agarose gel (w:v in PBS) at 1 cm around a central well containing 100 µL of normal human serum (NHS) (Etablissement Français du Sang, Brest, France) diluted 1:20 in PBS. The gels were incubated 24 to 48 hrs in a humidified atmosphere at room temperature.

Determination of the HC_{50} of the NHS used

The HC_{50} represents the concentration which leads to 50% lysis of sheep erythrocytes. Antibody-sensitized sheep erythrocytes (AE) were first prepared by incubating sheep erythrocytes (Eurobio, Paris, France) with rabbit anti-sheep erythrocyte antibodies (Biomerieux, Paris, France) as described by Kazatchkine et al. 1985. NHS (normal human serum) (800 µL) at various concentrations in VBS (veronal buffer saline: 4 mM veronal, 0.15 mM NaCl, 0.15 mM Ca^{2+}, 0.5 mM Mg^{2+}, pH 7.3) were

incubated for 45 min at 37ºC with 200 µL of AE (1:20). Controls of 0% (L_0) and 100% (L_{100}) of lysis were obtained by incubating 800 µL of VBS with 200 µL AE diluted 1:20 in the same conditions. Then, 0.15 M NaCl solution (2 mL) was added in each tube except the L_{100} tube, where 2 mL of DDW was added. After centrifugation (15 min at 1000 x g), OD of supernatants was measured at 414 nm.

Evaluation of the haemolytic capacity of NHS at HC_{50}

The capacity of NHS to lyse AE via the classical pathway was assessed with various amounts of rituximab, Fab or Fab-Gal fragments. For the HC_{50} assay, 800 µL of NHS (1:100 dilution in VBS) were incubated with 0.1 to 2 nmol of Fab or Fab-Gal and 200 µL of AE at 10^8 cells/mL, for 45 min at 37ºC. After addition of 2 mL 0.15 M NaCl and centrifugation, the residual HC_{50} units of the supernatant were determined by measuring the OD at 414 nm.

Hemolytic assay to evaluate the activation of the classical complement pathway

Various amounts of rituximab, Fab or Fab-Gal fragments (0 to 0.5 nmol) were pre-incubated for 45 min at 37ºC with 15 µL of NHS diluted in VBS (1:20) (the dilution at which 90% of cell lysis occurred in our experimental conditions). Then, a mixture of 100 µL of C4-deficient serum (Ingen, Rungis, France) and 100 µL of AE (1:20) were added and incubated for another 45 min at 37ºC. The controls L_0 and L_{100} were obtained by incubating 400 µL of VBS with 100 µL AE (1:20) in the same conditions. After addition of 0.15 M NaCl buffer (2 mL) or DDW (2 mL) and centrifugation, the amount of released hemoglobin was assessed by measuring the OD of supernatants at 414 nm.

Flow cytometry analysis

FITC conjugation. Fab fragments of rituximab or Fab-Gal conjugates (1 mg in 1 mL of 0.1 M sodium bicarbonate buffer, pH 9) were mixed with 100 µL of fluorescein isothiocyanate (FITC) (Sigma, Saint Quentin Fallavier, France) (10 mg/mL in DMSO) for 1 hr at room temperature. The reaction was stopped by adding 500 µL of 1.5 M hydroxylamine buffer, pH 8.5.

Staining measurement. Daudi cells (10^5) in 100 µL 1% PBS-BSA were incubated for 30 min at room temperature with 5 µL of mouse IgG isotype antibody labelled with FITC (Beckman Coulter, Paris, France), 10 µg of FITC-Fab or 10 µg of FITC-Fab-Gal conjugate. After two washes with 1% PBS/BSA and resuspension in PBS, 5000 cells were analyzed in a flow cytometer (Beckman Coulter, France) equipped with a 500 mV argon laser and an excitation wavelength of 488 nm to measure the mean of fluorescence FITC intensity (FITC).

Fluorescence microscope analysis

Microscope slides were prepared by cytocentrifugation using a Shandon Cytospin 4 (800 rpm, 1 min) for each cell preparation (10^5 cells in 100 µL) previously examined in direct-staining flow cytometry. The slides were analyzed under a fluorescence microscope (Olympus BX60) equipped with a digital camera with a magnification of 400.

Complement-dependant cellular cytotoxicity of the Fab-Gal conjugate

Daudi cells (obtained from a Burkitt's lymphoma patient, ATCC, Manassas, USA) (1.5 x 10^4) were incubated for 45 min at 37ºC under a humidified atmosphere with 1 nmol/mL of Fab fragments *versus* conjugated Fab-Gal fragments. Then, 15% of NHS (v:v) were added and the mixture was placed for 24 hrs at 37ºC under a humidified atmosphere at 5% CO_2 (v:v). After 12 hrs of incubation, 1 µL of tritiated thymidine (GE Healthcare Amersham Bioscience, Orsay, France) and 19 µL of RPMI-1640 were added and incubated for another 12 hrs. A negative control, lacking the antibody fragment, was prepared in the same conditions to measure spontaneous cell lysis. At the end of incubation, cell nuclei were transferred to glass fiber filter paper (Skatron instrument, Beckman Coulter, France) and placed in scintillation vials with 3 mL of scintillation fluid. The incorporation of tritiated thymidine was measured in a scintillation counter of β emissions (Packard, France) and results were expressed in dpm (number of disintegrations per minute).

DOI: 10.2225/vol15-issue5-fulltext-3

Statistical analysis

Comparisons were made using Fisher's test.

RESULTS

Recognition of seric immunoglobulins by the α-Gal epitope conjugated to Fab fragments

This recognition was evaluated by an Ouchterlony radial immunodiffusion (Ouchterlony, 1958) and resulted in the formation of immune complexes visualized on the gel by a precipitin band (Figure 2). On the migration path of the Normal Human Serum (NHS) (well 1) towards the Fab fragments of rituximab (well 2), no circular precipitation line was detected indicating that there were no seric antibodies against the Fab fragments of rituximab. In contrast, a precipitin line was visible between well 1 (NHS) and 3 (Fab-Gal, 35 µg) and between well 1 and 4 (Fab-Gal, 70 µg) confirming that seric immunoglobulins recognized the Fab-Gal bioconjugate.

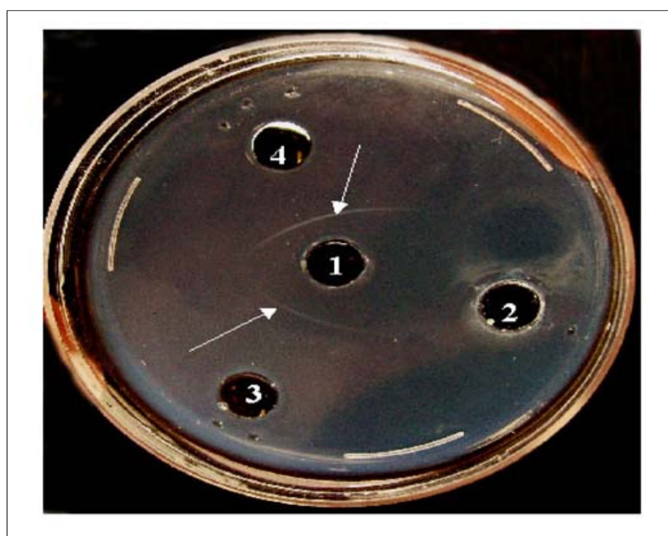

Fig. 2 Recognition by serum Igs of the xenoantigen conjugated to Fab fragments. Immunoprecipitation on 1% agarose. 100 µL of NHS (1:20) were placed in a central well. At approximately 1 cm from the central well, 1.4 nmol of each sample was loaded and the gel was incubated for 24 to 48 hrs under a humidified atmosphere at room temperature. Well 1: NHS. Well 2: rituximab Fab fragments. Well 3: Fab-Gal bioconjugate (35 µg). Well 4: Fab-Gal bioconjugate (70 µg). This is a representative example of the experiment performed in triplicate. White arrows show the precipitin lines.

Fab-Gal recognition of CD20 expression in Burkitt's lymphoma cells

Flow cytometry was used to evaluate the ability of Fab-Gal bioconjugates to recognize the CD20 epitope present at the Daudi cell surface. In an initial experiment, the Fab fragments and the Fab-Gal conjugates were linked to the FITC probe. These fluorescent compounds were then incubated with Daudi cells. As shown in Figure 3a, the histograms obtained for the Fab fragments and the Fab-Gal conjugate were comparable indicating that there were no major differences in the binding capacity of the Fab fragments and the conjugate Fab-Gal. The cell preparations assessed by flow cytometry were cytocentrifuged on microscope slides and observed under an Olympus BX60 fluorescence microscope with appropriate filters. The photographs in Figure 3b show similar cell fluorescence staining for both Fab fragments and Fab-Gal conjugates.

DOI: 10.2225/vol15-issue5-fulltext-3

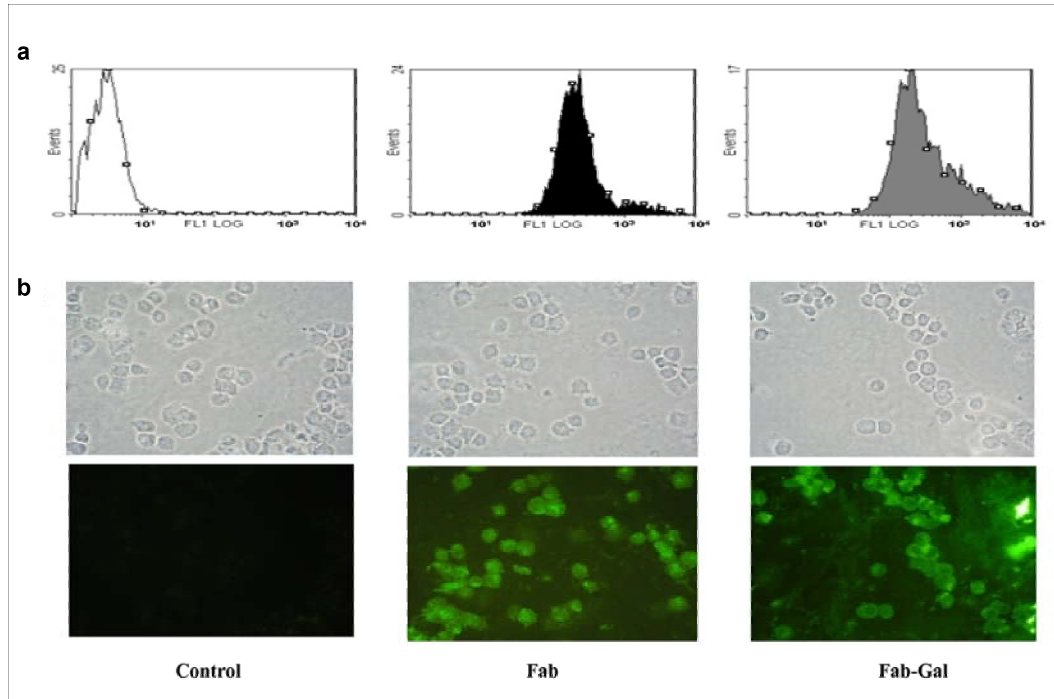

Fig. 3 Capacity of the Fab-Gal bioconjugates to recognize the CD20 epitope present at Daudi cell surfaces. Flow cytometry of binding of Fab fragments of rituximab and Fab-Gal bioconjugate conjugated to FITC with CD20 antigen present on Daudi cells. Daudi cells (10^5) were incubated for 30 min at room temperature with mouse IgG isotype antibody labeled with FITC (Control), FITC-Fab (Fab), or FITC-Fab-Gal conjugate (Fab-Gal). (a) Single-parameter histograms: x-axis, intensity of the fluorescence signal; y-axis, number of fluorescent cells. The intensity of fluorescence was measured using a FACScan cytometer. (b) Microscopic images from cell preparations observed under a fluorescence microscope (Olympus BX60) equipped with a digital camera and magnified 400x.

Effect of the Fab-Gal conjugate on complement activation and complement-dependent cytotoxicity of Burkitt's lymphoma cells in haemolytic assays

These assays made it possible to evaluate the activation of the complement in NHS. The assays were carried out in the presence of AE, which are the activators of the complement system and also the revealing agents for this reaction as, upon activation, they release haemoglobin which can be measured by spectrophotometry.

We used the following strategy to determine the activity of Fab-Gal on the complement system: we first determined the activity of Fab-Gal on the hemolytic capacity of NHS to lyse 50% of AE (HC_{50}). With this test, the inhibition or activation of the complement system could be observed. Then, we evaluated the capacity of Fab-Gal to restore the haemolytic activity of a C4-deficient serum.

In our experiments, HC_{50} was reached at a 1:100 dilution. In the first experiment, we worked with Fab fragments and Fab-Gal conjugates (2 nmol) either with or without pre-incubation to detect their effect on the haemolytic activity of the NHS. Figure 4a shows that NHS maintained its haemolytic capacity of 50% in the presence of either Fab fragments or the Fab-Gal conjugates. Therefore, these compounds did not have an inhibitory effect on the complement system.

To determine if these compounds were able to activate the complement system, a second experiment was performed in which the Fab fragments or the Fab-Gal conjugates were incubated with NHS (1:100) prior to the addition of AE. The results showed that the haemolytic capacity of NHS was not modified by incubation with the Fab fragments (Figure 4b), whereas NHS incubated with Fab-Gal conjugates showed a significant decrease in its haemolytic capacity.

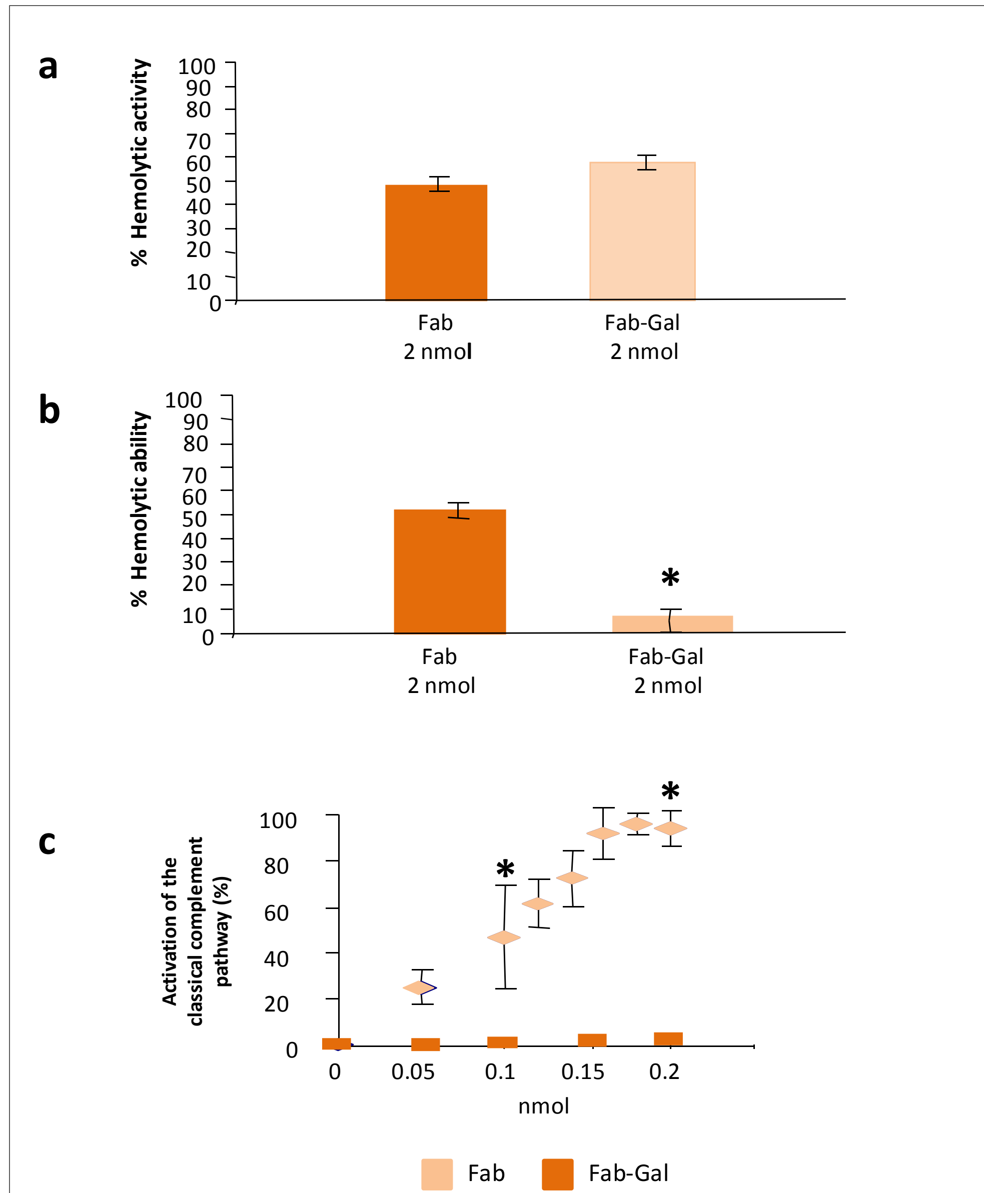

Fig. 4 Effect of Fab-Gal on complement activation. (a) 350 µl NHS at HC_{50} were incubated with 2 nmol Fab and Fab-Gal and 200 µl AE for 45 min at 37ºC. Controls 0% (L0) and 100% (L100) lysis (800 µL VBS buffer with 200 µL AE) were incubated in the same way. After the addition of 2 mL of 0.15M NaCl (or 2 mL DDW for L100) followed by centrifugation (15 min, 600 x g), OD of the supernatants was measured at 414 nm. This test was carried out three times in duplicate. Data are expressed as hemolytic ability calculated according to the following formula: % ability=100–[(ODSample-ODL0)/(ODControl 0 µg) x 100]. The ability of activating the classical complement pathway of Fab-Gal conjugates was compared with that of Fab fragments using a Student's *t*-test (*: $p < 0.05$). **(b)** Same experiment as in **(a)**, except 2 nmol of Fab and Fab-Gal were preincubated with NHS for 45 min at 37ºC before adding AE. This test was carried out three times in duplicate. The hemolytic ability of Fab-Gal and Fab were compared using a Student's *t*-test (*: $p < 0.05$). **(c)** Fab and Fab-Gal were incubated with 15 µL NHS for 45 min at 37ºC. Cell lysis by NHS was estimated using a negative control (0 nmol) under the same conditions. These mixtures were then incubated for 45 min at 37ºC with C4-deficient guinea pig serum (100 µL of 1:100 dilution), 100 µL AE and adjusted with VBS buffer to 500 µL. Controls 0% (L0) and 100% (L100) lysis (400 µL VBS buffer with 100 µL AE) were incubated in the same way. After adding 2 mL 0.15 M NaCl (or 2 mL DDW for L100) and centrifugation (15 min, 600 x g), OD of the supernatants was measured at 414 nm. Data are expressed as the percentage of activation calculated according to the following formula: % lysis=100-[(ODSample-ODL0)/(ODControl 0 µg)x100]. This test was carried out three times in duplicate. The ability of the Fab-Gal conjugates to activate the classical complement pathway was compared to that of Fab fragments using a Student's t-test (*: $p < 0.05$).

In a second experiment, using the restoration of the haemolytic capacity of a serum lacking an identified protein of the classical pathway of the complement system, we calculated the percentage of activation of the complement system according to concentration of Fab, Fab-Gal or rituximab. In these tests, performed with guinea pig serum deficient in the C4 complement protein, various concentrations of Fab fragments or Fab-Gal conjugates were first incubated with NHS and then the mixtures Fab-NHS and Fab-Gal-NHS were incubated with the C4-deficient guinea pig serum together with the AE. Figure 4c shows that Fab fragments had no effect on the complement system and confirms that Fab-Gal activates the classical complement pathway. With just 0.2 nmol of Fab-Gal conjugate, 90% of activation was reached.

Complement-dependent cytotoxicity of Fab-Gal on Burkitt's lymphoma cell line

To confirm the higher efficiency of Fab-Gal, the ability of the compounds to inhibit the proliferation of Daudi cell line was evaluated using a radioactive thymidine incorporation assay in dividing cells. The results, presented in Figure 5, show that the Fab fragments did not inhibit the proliferation of Daudi cells. In contrast, the Fab-Gal conjugates inhibited cellular proliferation by more than 50% only in presence of normal human serum confirming the activation of complement by the presence of α Gal epitope.

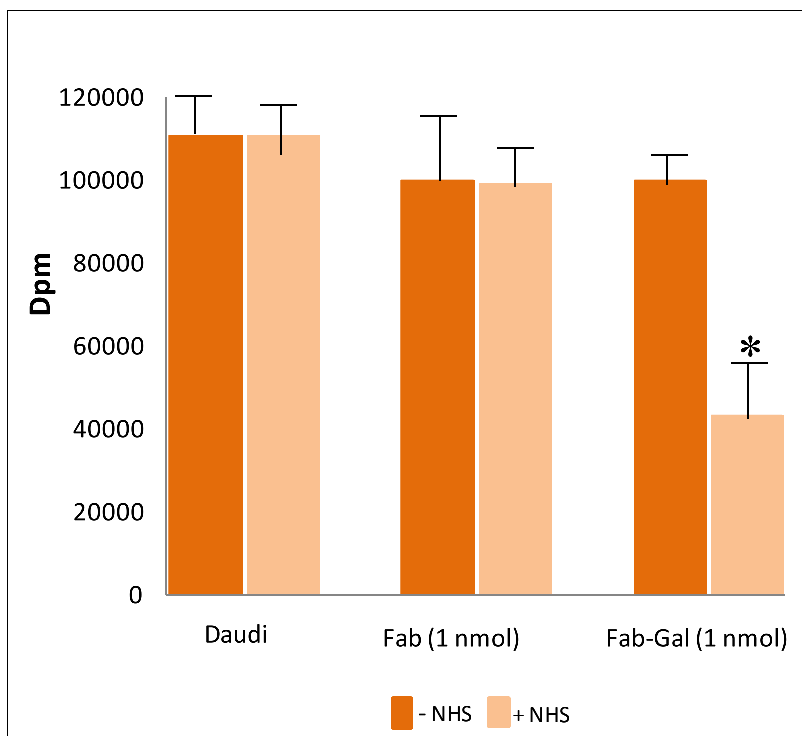

Fig. 5 Effect of Fab-Gal on Burkitt's lymphoma cell line complement dependent cytotoxicity. Evaluation of Daudi cell proliferation by [^{3}H]-thymidine incorporation in the presence of NHS (darker, control), NHS and Fab fragments (1 nmol/mL,) or Fab-Gal bioconjugate (1 nmol/mL). 1.5 x 10^4 cells were incubated with Fab fragments versus Fab-Gal for 45 min at 37ºC under a humidified atmosphere. Then 15% NHS or not was added and the incubation continued for 24 hrs at 37ºC. 12 hrs before the end of incubation, 1 µL [^{3}H]-thymidine and 19 µL RPMI 1640 were added. A negative control without samples (darker), obtained under the same conditions, provided a measurement of spontaneous cell lysis. Cell nuclei were recovered on glass fiber filter paper and transferred to scintillation vials containing scintillation fluid (5 mL) and radioactivity was determined using a liquid scintillation counter for 5 min. This test was carried out three times in triplicate. Mean values obtained with the Fab-Gal conjugate were compared to the mean obtained with Fab fragments using a Student's *t*-test (*: $p < 0.05$).

DISCUSSION

The aim of this study was to evaluate whether the covalent coupling of a highly immunogenic epitope could confer the ability to activate the complement system to Fab fragments. In order to illustrate this strategy, we chose a highly immunogenic oligosaccharide and Fab fragments obtained from a therapeutic monoclonal antibody. The α-Gal epitope is a key element in xenotransplantation rejection, especially in pig-to-human transplants, due to the recognition of this epitope primarily by natural IgMs that activate the classical pathway of the complement system. The use of a vector, such as rituximab Fab fragments that do not exhibit any activity on the complement system and that are not internalized by the cell, should lead to activation of the immune system in the area surrounding tumour cells. Haemolytic assays showed that intact rituximab as well as Fab fragments did not have any effect on the complement system, whereas Fab-Gal conjugates activated the classical pathway of the complement system. These results indicate that the Fab-Gal conjugate was recognized by natural IgGs, especially IgMs, present in NHS, leading to the formation of immune complexes owing to the bivalence of the epitopes grafted onto the Fab molecule. This recognition strongly activates the classical pathway of the complement system and therefore consumes the complement proteins present in the sera. Thus, we demonstrated that the α-Gal epitope can be conjugated to the Fab fragments of rituximab, and that it is recognized by seric Igs which activate the complement system. These results confirm the hypothesis that these seric Igs are essentially natural IgMs specific to the α-Gal epitope, which have a high capacity for activating the classical pathway of the complement system.

Some *in vitro* assays demonstrated that the Fab-Gal conjugate was able to induce complement-dependant cellular cytotoxicity.

Conjugation was performed using a chemical method with a heterobifunctional linker, which allowed specific reactions between amine functional groups on the glycan molecule with thiol groups on the antibody fragments. This type of linker avoids cross linking between polyfunctional molecules. In a more general case, a linker means any reactive molecule having at least one reactive functional group capable of forming a bond with a reactive function of the immunogenic molecule and at least one reactive functional group able of forming a bond with at least one reactive function of therapeutic antibody. A reactive functional group means any reactive function able of bonding covalently with another functional group, including functions such as amino, thiol, hydroxyl, carbonyl, ester activated hydrazine, isocyanate (Brinkley, 1992; Annunziato et al. 1993; Thompson, 2004; Xiao et al. 2004). In our case, we controlled the number of thiol groups generated, and for that purpose, the Fab fragments were mildly functionalized to generate an average of two thiol groups that can react with the heterobifunctional linker. A low level of functionalization was chosen to maintain a high capacity for recognizing the antigen present at the cells surface, here, CD20 while ensuring proper orientation of the α-Gal motif for satisfactory detection by the anti-Gal Igs in human serum. The bio conjugation could be carefully monitored using various methods. It makes possible to control the number of molecules linked to the mAb in function of the immunogenic activity required and of the state of patients.

CONCLUDING REMARKS

This strategy could be optimized and extrapolated to many therapeutic antibodies lacking either ADCC or CDC mechanisms by modulating the number, the nature and the immunogenicity level of the grafted oligosaccharide. For instance, a patient in aplasia presents a complement skill but not ADCC, an increases activity of mAb could be made by adjunction of molecule complement efficient, patients who present Natural killer FCg receptor genotypes for ADCC, could receive antibody treatment coupling with molecules that promote ADCC activity. In conclusion, this strategy could lead to generate immunotherapy more effective against tumours.

ACKNOWLEDGMENTS

AC and SGC realized the experiments and contributed to the interpretation and the writing of this manuscript. CB and JG are implicated in the conception of this study. AB and CB conceived the project, realized the interpretation of the results and wrote the article. The authors thank Roche (France) for supplying rituximab.

DOI: 10.2225/vol15-issue5-fulltext-3

Financial support: This work was supported by Brest Metropole Oceane, the association “Céline et Stéphane” and Cancéropôle Grand Ouest.

REFERENCES

ABDEL-MOTAL, U.M.; WIGGLESWORTH, K. and GALILI, U. (2009). Intratumoral injection of α-gal glycolipids induces a protective anti-tumor T cell response which overcomes Treg activity. *Cancer Immunology, Immunotherapy*, vol. 58, no. 10, p. 1545-1556. [CrossRef]

ALDOSS, I.T.; WEISENBURGER, D.D.; FU, K.; CHAN, W.C.; VOSE, J.M.; BIERMAN, P.J.; BOCIEK, R.G. and ARMITAGE, J.O. (2008). Adult Burkitt lymphoma: Advances in diagnosis and treatment. *Oncology (Williston Park)*, vol. 22, no. 13, p. 1508-1517.

ANNUNZIATO, M.E.; PATEL, U.S.; RANADE, M. and PALUMBO, P.S. (1993). *p*-Maleimidophenyl isocyanate: A novel heterobifunctional linker for hydroxyl to thiol coupling. *Bioconjugate Chemistry,* vol. 4, no. 3, p. 212-218. [CrossRef]

BEUM, P.V.; LINDORFER, M.A.; BEURSKENS, F.; STUKENBERG, P.T.; LOKHORST, H.M.; PAWLUCZKOWYCZ, A.W.; PARREN, P.W.; VAN DE WINKEL, J.G. and TAYLOR, R.P. (2008). Complement activation on B lymphocytes opsonized with rituximab or ofatumumab produces substantial changes in membrane structure preceding cell lysis. *The Journal of Immunology*, vol. 181, no. 1, p. 822-832.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, vol. 71, no. 1-2, p. 248-254. [CrossRef]

BRINKLEY, M. (1992). A brief survey of methods for preparing protein conjugates with dyes, haptens, and crosslinking reagents. *Bioconjugate Chemistry*, vol. 3, no. 1, p. 2-13. [CrossRef]

CARDARELLI, P.M.; QUINN, M.; BUCKMAN, D.; FANG, Y.; COLCHER, D.; KING, D.J.; BEBBINGTON, C. and YARRANTON, G. (2002). Binding to CD20 by anti-B1 antibody or F(ab')2 is sufficient for induction of apoptosis in B-cell lines. *Cancer Immunology, Immunotherapy*, vol. 51, no. 1, p. 15-24. [CrossRef]

CHOLUJOVA, D.; JAKUBIKOVA, J. and SEDLAK, J. (2009). BioBran-augmented maturation of human monocyte-derived dendritic cells. *Neoplasma*, vol. 56, no. 2, p. 89-95. [CrossRef]

COURTOIS, A.; SIMON-COLIN, C.; BOISSET, C.; BERTHOU, C.; DESLANDES, E.; GUÉZENNEC, J. and BORDRON, A. (2008). Floridoside extracted from the red alga *Mastocarpus stellatus* is a potent activator of the classical complement pathway. *Marine Drugs*, vol. 6, no. 3, p. 407-417. [CrossRef]

EDWARDS, J.C.; LEANDRO, M.J. and CAMBRIDGE, G. (2002). B-lymphocyte depletion therapy in rheumatoid arthritis and other autoimmune disorders. *Biochemical Society Transactions*, vol. 30, no. 4, p. 824-828. [CrossRef]

EISENBERG, R. (2003). SLE - Rituximab in lupus. *Arthritis Research & Therapy*, vol. 5, no. 4, p. 157-159. [CrossRef]

ELLMAN, G.L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics*, vol. 82, no. 1, p. 70-77. [CrossRef]

GALILI, U.; SHOHET, S.B.; KOBRIN, E.; STULTS, C.L. and MACHER, B.A. (1988). Man, apes, and Old World monkeys differ from other mammals in the expression of alpha-galactosyl epitopes on nucleated cells. *The Journal of Biological Chemistry*, vol. 263, no. 33, p. 17755-17762.

GALILI, U. and LATEMPLE, D.C. (1997). Natural anti-Gal antibody as a universal augmenter of autologous tumor vaccine immunogenicity. *Immunology Today*, vol. 18, no. 6, p. 281-285. [CrossRef]

GALILI, U. (2001). The α-gal epitope (Galα1-3Galβ1-4GlcNAc-R) in xenotransplantation. *Biochimie*, vol. 83, no. 7, p. 557-563. [CrossRef]

GALILI, U.; CHEN, Z.C. and DEGEEST, K. (2003). Expression of α-gal epitopes on ovarian carcinoma membranes to be used as a novel autologous tumor vaccine. *Gynecologic Oncology*, vol. 90, no. 1, p. 100-108. [CrossRef]

GOTTARDO, N.G.; BAKER, D.L. and WILLIS, F.R. (2003). Successful induction and maintenance of long-term remission in a child with chronic relapsing autoimmune hemolytic anemia using rituximab. *Pediatric Hematology-Oncology*, vol. 20, no. 7, p. 557-561. [CrossRef]

JUHL, H.; PETRELLA, E.C.; CHEUNG, N.K.; BREDEHORST, R. and VOGEL, C.W. (1990). Complement killing of human neuroblastoma cells: A cytotoxic monoclonal antibody and its $F(ab)'_2$-cobra venom factor conjugate are equally cytotoxic. *Molecular Immunology*, vol. 27, no. 10, p. 957-964. [CrossRef]

KAZATCHKINE, M.; HAUPTMANN, G. and NYDEGGER, U. (1985). Dosages hémolytiques des composants du Complément. In: *Techniques du Complément, Techniques en Immunologie*. INSERM, Paris: Société Française d'Immunologie. p. 15-44.

LATEMPLE, D.C.; HENION, T.R.; ANARAKI, F. and GALILI, U. (1996). Synthesis of α-galactosyl epitopes by recombinant α1,3galactosyltransferase for opsonization of human tumor cell vaccines by anti-galactose. *Cancer Research*, vol. 56, no. 13, no. 3069-3074.

MANCHES, O.; LUI, G.; CHAPEROT, L.; GRESSIN, R.; MOLENS, J.P.; JACOB, M.C.; SOTTO, J.J.; LEROUX, D.; BENSA, J.C. and PLUMAS, J. (2003). *In vitro* mechanisms of action of rituximab on primary non-Hodgkin lymphomas. *Blood*, vol. 101, no. 3, p. 949-954. [CrossRef]

MCMORROW, I.M.; COMRACK, C.A.; SACHS, D.H. and DERSIMONIAN, H. (1997). Heterogeneity of human anti-pig natural antibodies cross-reactive with the gal([alpha]1,3)galactose epitope 1. *Transplantation*, vol. 64, no. 3, p. 501-510.

OUCHTERLONY, Ö. (1958). Diffusion-in-gel methods for immunological analysis. *Progress in Allergy*, vol. 5, p. 1-78. [CrossRef]

REITER, Y. and FISHELSON, Z. (1989). Targeting of complement to tumor cells by heteroconjugates composed of antibodies and of the complement component C3b. *The Journal of Immunology*, vol. 142, no. 8, p. 2771-2777.

SANDRIN, M.S.; VAUGHAN, H.A.; DABKOWSKI, P.L. and MCKENZIE, I.F. (1993). Anti-pig IgM antibodies in human serum react predominantly with Gal(alpha 1-3)Gal epitopes. *Proceedings of the National Academy Sciences of the United States of America*, vol. 90, no. 23, p. 11391-11395.

SEHN, L.H. (2008). Management of Burkitt lymphoma: A continuing challenge. *Oncology (Williston Park)*, vol. 22, no. 13, p. 1519-1522-3.

SHAN, D.; LEDBETTER, J.A. and PRESS, O.W. (2000). Signaling events involved in anti-CD20-induced apoptosis of malignant human B cells. *Cancer Immunology, Immunotherapy*, vol. 48, no. 12, p. 673-683. **[CrossRef]**

THOMPSON, S. (2004). Small-molecule-protein conjugation procedures. *Molecular Diagnosis of Infectious Diseases, Methods in Molecular Medicine*, vol. 94, p. 255-265. **[CrossRef]**

VAN MEERTEN, T.; VAN RIJN, R.S.; HOL, S.; HAGENBEEK, A. and EBELING, S.B. (2006). Complement-induced cell death by rituximab depends on CD20 expression level and acts complementary to antibody-dependent cellular cytotoxicity. *Clinical Cancer Research*, vol. 12, no. 13, p. 4027-4035. **[CrossRef]**

WALPEN, A.J.; MOHACSI, P.; FREY, C.; ROOS, A.; DAHA, M.R. and RIEBEN, R. (2002). Activation of complement pathways in xenotransplantation: An *in vitro* study. *Transplant Immunology*, vol. 9, no. 2-4, p. 271-280. **[CrossRef]**

WANG, Y.; FEI, D.; VANDERLAAN, M. and SONG, A. (2004). Biological activity of bevacizumab, a humanized anti-VEGF antibody *in vitro. Angiogenesis*, vol. 7, no. 4, p. 335-345. **[CrossRef]**

WANG, S.Y.; RACILA, E.; TAYLOR, R.P. and WEINER, G.J. (2008). NK-cell activation and antibody-dependent cellular cytotoxicity induced by rituximab-coated target cells is inhibited by the C_3b component of complement. *Blood*, vol. 111, no. 3, p. 1456-1463. **[CrossRef]**

XIAO, S.J.; BRUNNER, S. and WIELAND, M. (2004). Reactions of surface amines with heterobifunctional cross-linkers bearing both succinimidyl ester and maleimide for grafting biomolecules. *The Journal of Physical Chemistry B*, vol. 108, no. 42, p. 16508-16517. **[CrossRef]**

ZECCA, M.; NOBILI, B.; RAMENGHI, U.; PERROTTA, S.; AMENDOLA, G.; ROSITO, P.; JANKOVIC, M.; PIERANI, P.; DE STEFANO, P.; BONORA, M.R. and LOCATELLI, F. (2003). Rituximab for the treatment of refractory autoimmune hemolytic anemia in children. *Blood*, vol. 101, no. 10, p. 3857-3861. **[CrossRef]**

ZHENG, X.; PALLERA, A.M.; GOODNOUGH, L.T.; SADLER, J.E. and BLINDER, M.A. (2003). Remission of chronic thrombotic thrombocytopenic purpura after treatment with cyclophosphamide and rituximab. *Annals of Internal Medicine*, vol. 138, no. 2, p. 105-108.

How to reference this article:

COURTOIS, A.; GAC-BRETON, S.; BERTHOU, C.; GUÉZENNEC, J.; BORDRON, A. and BOISSET, C. (2012). Complement dependent cytotoxicity activity of therapeutic antibody fragments is acquired by immunogenic glycan coupling. *Electronic Journal of Biotechnology*, vol. 15, no. 5. http://dx.doi.org/10.2225/vol15-issue5-fulltext-3

Note: Electronic Journal of Biotechnology is not responsible if on-line references cited on manuscripts are not available any more after the date of publication. Supported by UNESCO / MIRCEN network.