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ORIGINAL ARTICLE

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Development of a new vaccine formulation that enhances the immunogenicity of tumor-associated antigen CaMBr1

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Abstract Aberrant glycosylation is one of the most constant traits of malignant cells. The CaMBr1 hexasaccharide antigen, originally defined on the human breast carcinoma cell line MCF7, is expressed on some normal tissues but overexpressed in a high percentage of human breast, ovary, prostate and lung carcinomas. CaMBr1 overexpression is associated with poor prognosis. The epitope consists of the tetrasaccharide Fuc(α1-2)Gal(β1-3)GalNAc(β1-3)Galα-*O*-spacer, which has recently become available as a synthetic oligosaccharide. Here we report the CaMBr1 tetrasaccharide conjugation to two different carrier proteins (CRM197 and KLH) and the evaluation of conjugate immunogenicity in mice following their administration in various vaccine formulations with two adjuvants (MPL-SE and Detox-PC). Radioimmunoassay to determine the level and isotype of anti-tetrasaccharide antibodies in mouse sera, and cytofluorimetric analysis and ^{51}Cr-release assay on human tumor cells, to evaluate specificity of binding and complement-dependent lysis respectively, identified CaMBr1-CRM197, in association with the MPL-SE adjuvant, as the best vaccine formulation. This combination induced (1) production of tetrasaccharide-specific antibodies, with negligible side-effects; (2) antibodies with complement-mediated cytotoxic activity on human CaMBr1-positive cells and (3) a high titer of IgG1 detected in sera obtained 3 months after the first injection, indicating that the anti-tetrasaccharide antibody response was mediated by T cell activation. The availability of CaMBr1-glycoconjugate in the minimal and functional antigenic structure and the identification of an efficacious vaccine formulation opens the way to exploring the activity of this glycoconjugate in a clinical setting.

Key words Tumor-associated-antigen · Immunogenicity · Vaccine formulation · Glycoconjugates

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Introduction

Malignant cells are often characterized by aberrant glycosylation patterns. A significant part of a cancer patient's humoral response is directed against cell-surface carbohydrate antigens such as gangliosides, glycolipids and glycoproteins [29, 39].

Different approaches have been adopted to induce an immune response against tumor-associated antigens (TAA), including vaccination with tumor cells and purified or synthetic carbohydrates [32, 35]. Because saccharides appear to be recognized mainly by T-cell-independent mechanisms, there is no efficient production of antibodies at significant levels or with high affinity; furthermore there is no induction of T cell memory required for booster responses. The immunogenicity of saccharidic TAA has been explored in a series of clinical trials, using hapten carrier-protein conjugates to induce reponses against non-immunogenic compounds [1, 18, 22].

Complement-mediated lysis and antibody-dependent cellular cytotoxicity are both efficient mechanisms mediating the antibody response against circulating tumor cells and micrometastases [14, 36]. Although the relative contribution of these mechanisms is still unclear, vaccines containing carbohydrate antigens, including Thomsen Friedenreich (TF) [1] and sialylated Tn (sTn) [37] or gangliosides [19–21], were introduced in phase

I–II clinical trials and shown to induce antibodies against these antigens in patients.

CaMBr1 is a blood-group-related TAA, originally identified on the human breast carcinoma cell line MCF7 as being identical to the Globo-H antigen of teratocarcinoma, using the murine monoclonal antibody (mAb) MBr1 [4, 5]. The epitope, carried on both glycolipids and glycoproteins [25], consists of the tetrasaccharide Fuc(α1-2)Gal(β1-3)GalNAc(β1-3)Galα-*O*-spacer [2]. Like most other human TAA, CaMBr1 is expressed on some normal tissues [9, 24] but overexpressed in a high percentage of human breast, ovary, prostate and lung carcinomas. Such overexpression is associated with poor prognosis [25, 28]. These characteristics led to efforts to target the CaMBr1 antigen for immune stimulation, and the immunogenicity of the fully synthetic Globo-H hexasaccharide has been recently demonstrated in a clinical trial [34].

In the present study, we explored the immunogenicity of the minimal defined antigenic structure of CaMBr1, now available by synthesis [2], in a preclinical mouse model. First we demonstrated that the carrier-protein conjugation method allows the production of glycoconjugates, which maintain the epitope conformation of the tetrasaccharide. Subsequently we focused on defining optimal adjuvant use, carrier protein specificity, and vaccination dose. Analysis of the specificity of the antibody response after immunization with CaMBr1 conjugated with the carrier proteins CRM197 or keyhole limpet hemocyanin (KLH) revealed a tetrasaccharide-specific production of IgM and IgG1 antibodies able to induce complement-mediated cytotoxicity of CaMBr1-positive cells. Thus, induction of T cell memory enables the switch to the IgG isotype of the antibody response.

Materials and methods

Cell lines and monoclonal antibodies

Human breast carcinoma cell line MCF7, which overexpresses CaMBr1, was obtained from American Type Culture Collection (ATCC, Rockville, Md., USA).

The following human CaMBr1-negative cell lines were used as negative controls: vulval carcinoma A431 (ATCC) and ovarian carcinomas SKOV3 (ATCC), OVCAR3 (Dr. Ira Pastan, NIH, Bethesda, Md., USA), SW626 (Memorial Sloan-Kettering Cancer Center, N.Y., USA), OVCA432 (Dr. Knapp, Dana Farber Institute, Boston, Mass., USA) and IGROV1 (Dr. Bernard, Institute G. Roussy, Villejuif, France). All cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 1% glutamine and 100 μg/ml gentamicin; cells were grown as monolayers and harvested by trypsin treatment.

Murine mAb MBr1 (IgM), derived from a Balb/c mouse immunized with MCF7 human breast carcinoma cells as described [27], is directed to the terminal tetrasaccharide sequence of Globo-H [4, 5]. MBr1 was purified from ascitic fluid by affinity chromatography using the anti-MBr1-idiotype mAb A3B10 (IgG1) linked to an agarose-bead support (Pierce, Rockford, Ill., USA).

Synthesis of the CRM197- and KLH-tetrasaccharide conjugates

The tetrasaccharide Fuc*p*-α-(1→2)-Gal*p*-β-(1→3)-Gal*p*NAc-β-(1→3)-Gal*p*-α-(1→*O*)-allyl (**9**, Fig. 1) has been synthesized essentially by our previously published procedure [17] with some modifications. The synthesis of **9**, as well as its conjugation to the immunogenic proteins KLH and CRM197, will be described in detail elsewhere.

Briefly, the protected galactose **2** was glycosylated with oxazoline **1** in the presence of triflurometh anesulfonic acid (TfOH) to give the disaccharide **3** in 70% yield (see Fig. 1). The glucosamine moiety of the disaccharide **3**, was transformed into the corresponding galactosamine to give **4** in 79% yield. Glycosylation of **4** with the donor **5**, again using TfOH as promoter, gave the trisaccharide **6** in a satisfactory 84% yield. A change of the protecting groups by Zemplén deacylation followed by benzylidene formation with 1,1-dimethoxytoluene and camphorsulfonic acid, according to our previously published procedure [16], gave **7** in 92% yield. The final glycosylation was performed using the donor **8**, again with TfOH as promoter, to give the tetrasaccharide **9** in 81% yield.

The tetrasaccharide **9** was then elaborated at the allylic moiety in order to introduce a proper spacer for the protein conjugation to each of the two carrier proteins. As carriers we used KLH [38], purchased from Cal-Biochem (La Jolla, Calif., USA), and CRM197, a non-toxic mutant of diphtheria toxin purified from cultures of *Corynebacterium diphtheriae*, kindly provided by Chiron Vaccines (Siena, Italy).

The strategy followed for the formation of the linkage with the protein was the introduction of a reactive monohydroxysuccinimidyl ester of an adipic acid spacer. Thus, the double bond of the allyl group of **9** was cleaved by treatment with $OsO_4/NaIO_4$ and the aldehyde group obtained was directly oxidized with $NaClO_2$ and esterified with diazomethane to give the glycosyl derivative of methyl glycolate **11**. Treatment of **11** with an excess of ethylendiamine gave the compound **12** in 92% yield. At this stage of the synthesis, the benzyl and benzylidene protecting groups were removed by catalytic hydrogenolysis with H_2-$Pd(OH)_2/C$ in the presence of acetic acid in almost quantitative yield.

The deprotected disaccharide **13** was treated with an excess of bis-hyroxysuccinimidyl adipate to give the derivative **14**. After the elimination of the excess reagent, compound **14** was used directly for the protein conjugation because of the lability of the activated ester function. An aliquot of compound **14** and the protein (KLH or CRM197) were dissolved in phosphate buffer (pH 7.4) and stirred overnight at room temperature. The solution was then lyophilized and the solid dialyzed to give the conjugates **15a** and **15b**.

Immunogen characterization

Tetrasaccharide:protein ratios were determined by trinitrobenzenesulfonic acid assay [11].

The ability of the glycoconjugates to inhibit MBr1 binding on target cells was evaluated by cytofluorimetry. MCF7 cells (4×10^5/sample) were incubated with serial dilutions (200 μM, 20 μM and 2 μM) of glycoconjugates or tetrasaccharide alone, in association with 5 μg/ml MBr1, for 30 min on ice. After three washes with phosphate-buffered saline (PBS) containing 0.03% bovine serum albumin (BSA), cells were further incubated with fluorescein-isothiocyanate (FITC)-labeled goat anti-(mouse Ig G + M) (Kierkegaard & Perry Laboratories Inc. Gaithersburg, Md.) for 30 min on ice. MBr1 binding was detected by flow cytometry (FACScan; Becton Dickinson, San Josè, Calif.). IC_{50}, extrapolated from dose/effect curves, is the saccharide concentration able to inhibit binding of mAb MBr1 by 50%.

Adjuvants

MPL-SE (monophosphoryl lipid A), a less toxic component derivative of lipopolysaccharide, is a squalene(2.5%)-in-water emulsion [6, 33]. Detox-PC is a lyophilized squalene(1%)-in-water emulsion containing MPL-SE and the cell wall skeleton from *Mycobacterium phlei* [23] Both MPL-SE and Detox-PC formulations were obtained from RIBI ImmunoChem Research Inc. (Hamilton, Montana, USA).

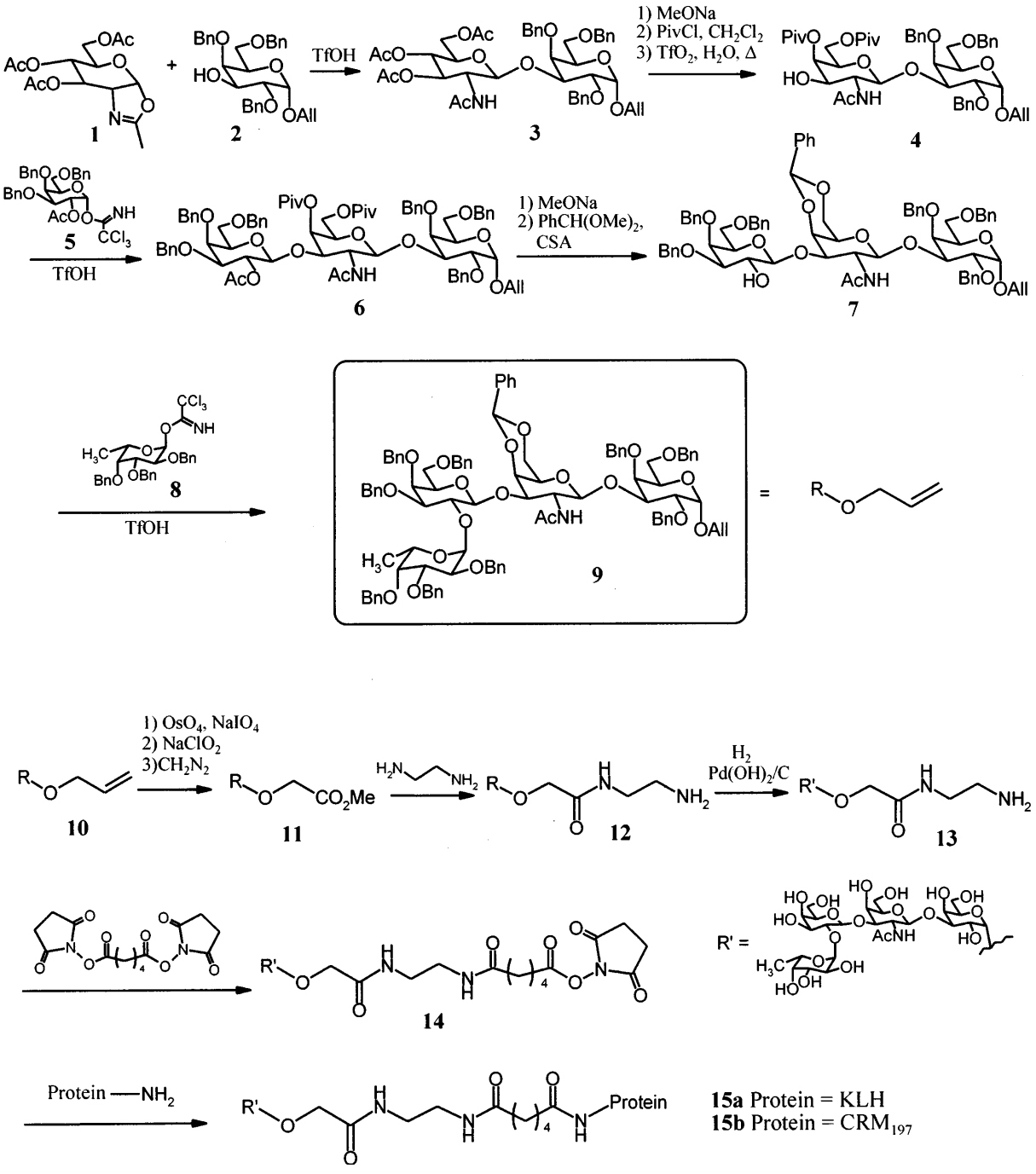

Fig. 1 Scheme for the synthesis of the tetrasaccharide and its glycoconjugates. *Ph* phenyl, *Bn* benzyl, *Piv* 2,2-dimethylpropionyl(pivaloyl), *Tf* trifluoromethanesulfonyl, *All* allyl

Animals and vaccine administration

Six-week-old female Balb/c × C57BL/6 mice (Charles River, Calco, LC, Italy) were injected subcutaneously (five mice/group) with CRM197- or KLH-tetrasaccharide glycoconjugate, using different doses of tetrasaccharide (0.5, 2.5 and 12.5 μg) and MPL-SE (final dilution 1:4) or Detox-PC (final dilution 1:10), given at two separate sites for a total volume of 200 μl [13, 37]. Control mice were injected with adjuvant alone. Mice were immunized on days 0, 14, 28 and boosted on days 49 and 129. Immune sera were collected on days 21, 35, 57, 120 and 136. Control sera were obtained from mice 1 week before the first vaccine injection.

Serological assays

Serum absorption

CaMBr1-negative cell lines (SKOV3, OVCAR3, SW626, OVCA432 and IGROV1) were harvested and pooled. Cells (5×10^6) were washed twice with 0.03% BSA in PBS and resuspended in 100 μl 0.03% BSA in PBS containing 1:5 pre- or post-vaccination sera. Samples were incubated for 1 h at 37 °C, with slow shaking. Sera were collected by centifugation at 2000 rpm for 10 min and stored at −20 °C.

RIA for anti-tetrasaccharide antibodies

The development of an antibody response specifically directed against the carbohydrate moiety was measured by quantitative radioimmuoassay (RIA) on purified and carrier-protein-conjugated tetrasaccharide, not used for vaccination (i.e. KLH-immunized animals were tested on CRM197-tetrasaccharide and vice versa). Microtiter 96-well plates (Greiner) were coated overnight at 4 °C with 0.5 μg (protein moiety) glycoconjugates at 50 μl/well (diluted in PBS) and saturated for 2 h at 37 °C with 100 μl PBS containing 2% BSA. After washing, the antigen-coated plates were incubated for 1 h at room temperature with 100-μl samples of mouse serum at different dilutions. Antibody binding was detected by further incubation for 45 min at 37 °C with sheep ^{125}I-labeled anti-(mouse Ig) (Amersham, Little Chalfont, UK) at 10^5 cpm/well and evaluated using a γ-counter (Crystal II Multidetector RIA System). Wells treated with sheep ^{125}I-labeled anti-(mouse Ig) only served as negative controls.

The Ig isotype was determined by RIA as described above, using subclass-specific secondary biotinylated goat anti-mouse IgG1, -IgG2a, -IgG2b mAb (Amersham) followed by incubation with ^{125}I-labeled streptavidin (Amersham, Little Chalfont, UK) (10^5 cpm/well).

The titer was defined as the serum dilution at which binding levels three times higher than the background were still detectable.

FACS analysis

MCF7 and A431 cell suspensions were washed three times with PBS containing 0.03% BSA. Cells (4×10^5/sample) were incubated with 100 μl 1:20 and 1:100 diluted pre- or post-immunization serum for 30 min on ice. After washing, 1:100 diluted secondary biotinylated anti-(mouse Ig G + M) mAb (Amersham) were added for 30 min on ice. Cells were washed as above and incubated with 1:200 diluted FITC-labeled streptavidin (Amersham), resuspended in 500 μl 0.03% BSA in PBS, and analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, Calif.).

Cytotoxicity assay

Complement-dependent cytotoxicity was tested by ^{51}Cr-release assay [31]. MCF7 and A431 cells (10^6 each) were labeled with 100 μCi ^{51}Cr, seeded into each well of a 96-well V-bottom plate (5×10^3 cells/well) and incubated with different dilutions of pre- or post-vaccination sera or with medium alone for 45 min at 37 °C. Cells were washed and rabbit complement (diluted 1:40; Cederlane, Hornby, Ontario) was added for 2 h at 37 °C. Supernatants were collected by centrifugation, and radioactivity was evaluated by β-counter (Beckman).

All assays were performed in triplicate and included, as positive control, wells for maximum release in 0.05% Triton-X100. Spontaneous release was evaluated in the absence of complement. The percentage specific lysis was calculated as follows:

$$\text{Cytotoxicity}(\%) = 100 \times [\text{experimental release (cpm)} - \text{spontaneous release (cpm)}]/[\text{maximum release (cpm)} - \text{spontaneous release (cpm)}]$$

Results

Immunogen characterization

The modifications in the synthesis of the tetrasaccharide Fuc*p*-α-(1→2)-Gal*p*-β-(1→3)-Gal*p*NAc-β-(1→3)-Gal*p*-α-(1→*O*)-allyl **9** (see Fig. 1) improved the yields and reduced the number of synthetic steps. In particular, all the glycosylation reactions used TfOH as the promoter because, in our hands, this catalyst gave better yields than those achieved with BF_3 or trimethylsilyl trifluoromethanesulfonate.

Tetrasaccharide:protein ratios were 248:1 (2.4 μg tetrasaccharide:10 μg protein) and 11:1 (1.93 μg tetrasaccharide:10 μg protein). The tetrasaccharide conjugated to CRM197 or KLH carrier protein and the tetrasaccharide alone were compared for their ability to inhibit MBr1 binding on MCF7 target cells. As shown in Fig. 2, both the conjugated tetrasaccharides as well as the tetrasaccharide alone inhibited MBr1 binding in a dose-dependent manner and with comparable IC_{50} values. These data indicate that the conjugation process did not modify the conformation of the epitope.

Antibody response

Quantitative RIA revealed a specific anti-tetrasaccharide IgM and IgG response by day 35 in mice immunized with CRM197- or KLH-tetrasaccharide, in the presence of either MPL-SE or Detox-PC adjuvant. Vaccines containing adjuvant alone were not immunogenic (Fig. 3).

Adjuvant selection

MPL-SE and Detox-PC adjuvant formulations, used with the KLH-tetrasaccharide conjugate, were equally efficient as far as the timing of the induction of high-titer IgM and IgG was concerned (Fig. 3). Two different doses of KLH-tetrasaccharide conjugate were used and no significant differences were observed at each assay time (except in the case of the last bleed-

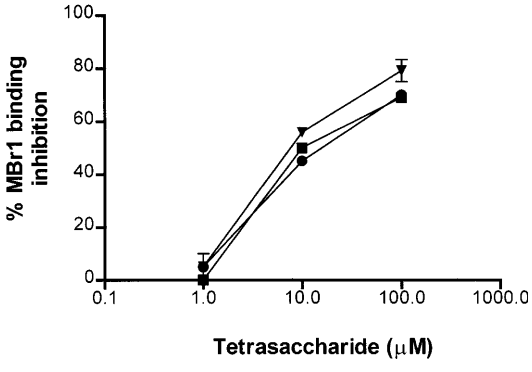

Fig. 2 Inhibition of mAb MBr1 binding by the synthetic glycoconjugates. MCF7 cells were incubated at 0 °C with a fixed amount of MBr1 (5 μg/ml) and serial dilutions (200 μM, 20 μM, 2 μM) of tetrasaccharide (▼), CRM197-tetrasaccharide (■), or keyhole-limpet-hemocyanin(KLH)-tetrasaccharide (●). Binding was evaluated by FACScan. Each value represents the mean (± SD) of three experiments

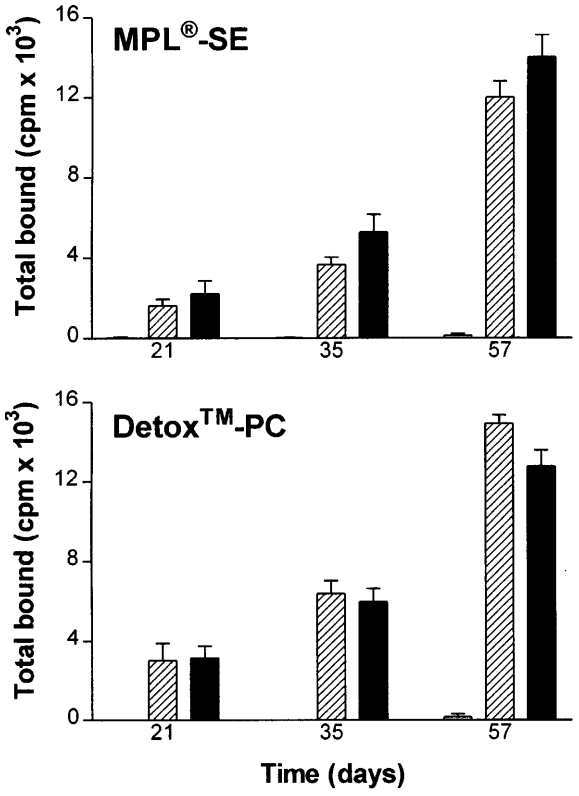

Fig. 3 MPL-SE and Detox-PC compared for adjuvant properties when used with KLH-tetrasaccharide. Binding activity in sera derived from mice injected with adjuvant alone (▨), with adjuvant plus KLH-tetrasaccharide (0.5 μg tetrasaccharide; ▨) or with KLH-tetrasaccharide (2.5 μg tetrasaccharide; ■). The antibody response was detected by quantitative radioimmunoassay (RIA) on the glycoconjugate not used for vaccination. Data are means (±SD) of values for sera from five different mice (1:500 diluted), collected at the indicated times after the first immunization. Binding values were compared by Student's *t*-test (unpaired, two-tailed) and differences were considered significant when *P* values were below 0.05 with a 95% confidence interval

ing, after the immunization with conjugate plus Detox-PC: $P = 0.0427$). The use of adjuvants was associated with adverse side-effects (granuloma at the injection sites), probably as a consequence of their composition. Because the toxicity (number and size of granulomas) was greater with Detox-PC only MPL-SE was used as adjuvant in further analysis of immunization protocols.

Dose effect

In keeping with the results reported in Fig. 3, no dose-dependent effect was observed with sera from mice injected with different doses of KLH-tetrasaccharide ($P = 0.1273$) whereas sera of mice vaccinated with 2.5 μg CRM197-tetrasaccharide showed a significantly higher level of antibody than those of mice receiving 0.5 μg ($P = 0.024$) or 12.5 μg ($P = 0.0467$) antigen (Fig. 4). MPL-SE was used as adjuvant in both cases.

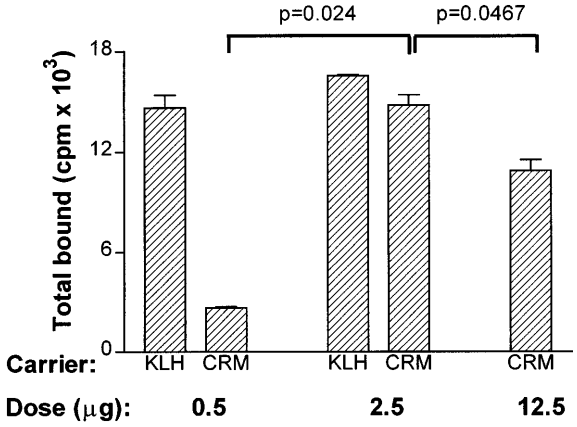

Fig. 4 Serum binding after immunization of mice with KLH- or CRM197-tetrasaccharide at different doses. Sera from two mice, obtained after the last injection (day 57), were pooled and diluted 1:500. Antibody binding to the glycoconjugate not used for vaccination was detected by quantitative RIA. Data are means (±SD) of values from three or four independent experiments. The binding values, at different doses, were compared by Student's *t*-test (unpaired, two-tailed) and differences were considered significant when *P* values were below 0.05 with a 95% confidence interval

Specificity of antibody response in mice immunized with KLH- or CRM197- CaMBr1 plus MPL-SE

To determine whether the CaMBr1 conjugates (2.5 μg) plus MPL-SE induced a specific antibody response, serum reactivity was tested by RIA on the purified tetrasaccharides before and after their absorption on CaMBr1-negative cell lines. Pre-immune sera showed reactivity only against KLH (Fig. 5A); the generic anti-KLH response was not increased by vaccination with the CRM197 protein (Fig. 5C) and was abrogated by pre-absorption. Serum absorption led to a significant decrease ($P = 0.0088$) in the total radioactivity bound in immune sera when KLH-tetrasaccharide was used for vaccination (Fig. 5B). By contrast, antibody reactivity before and after serum absorption on CaMBr1-negative human cell lines did not differ significantly when CRM197-tetrasaccharide was used for immunization (Fig. 5C). Total or pre-absorbed sera were also tested, by fluorescence-activated cell sorting analysis, on human CaMBr1-positive cells (MCF7) and on a CaMBr1-negative cell line (A431), not included in the absorption panel. Vaccination induced higher antibody binding to MCF7 cells than to A431 cells; serum absorption abolished the fluorescence observed on A431 cells, while serum reactivity on MCF7 cells was only marginally reduced (Fig. 6).

Preponderance of IgG1 in sera from immunized mice

Sera from 2 of 5 mice vaccinated with CaMBr1-CRM197, using MPL-SE as adjuvant, were pooled at

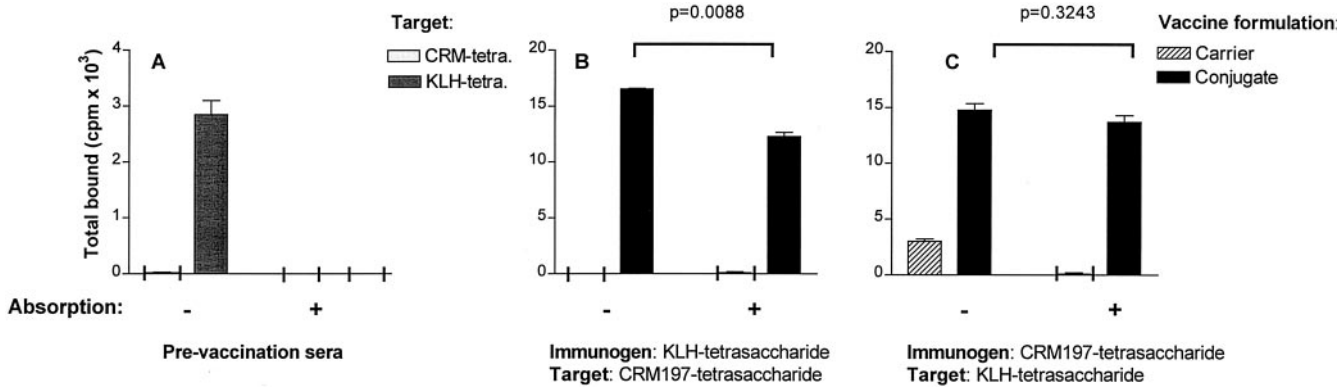

Fig. 5A–C Binding activity in sera of mice immunized with KLH-tetrasaccharide or CRM197-tetrasaccharide. Binding activity was assessed before and after absorption on CaMBr1-negative cell lines. Sera (1:500 diluted) were obtained before the first injection (**A**) and after the last administration of carrier protein alone (▨) or the complete vaccine formulation (■) of KLH-tetrasaccharide (**B**) or CRM197-tetrasaccharide (**C**). Antibody binding to the glycoconjugate not used for vaccination was detected by quantitative RIA

each assay time and tested for antibody isotype by RIA using a panel of IgG-subclass-specific secondary antibodies. After the first and second immunizations, all isotypes were equally represented but, after the third injection, serum IgG levels increased; in sera collected 10 weeks after the boost (day 57), serum IgG was mainly of the IgG1 subclass (Table 1). The titer of the different isotypes at this time, defined as the serum dilution at which binding levels were threefold higher than background, was IgM = 1:2000, IgG1 = 1:64 000, IgG2a = 1:16 000, IgG2b = 1:8000.

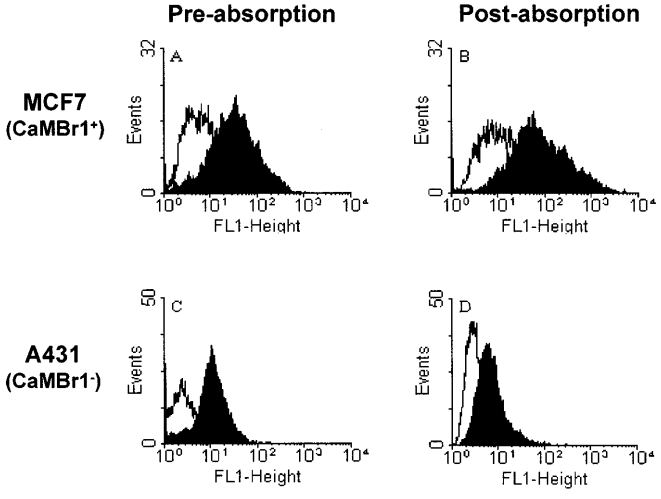

Fig. 6A–D Fluorescence-activated cell sorting analysis for specific anti-tetrasaccharide antibodies. Binding specificity of sera was evaluated before (**A**, **C**) and after (**B**, **D**) absorption on CaMBr1-negative cell lines. Reactivity was tested on MCF7 (**A**, **B**) and A431 (**C**, **D**) cells. Fluorescence intensity of pooled sera (1:100 diluted) (two sera/group) was assessed on day 57 (*black histogram*) or before vaccination (*outline histogram*)

Complement-mediated cytotoxicity after vaccination

To determine the possible effector function of anti-CaMBr1 antibodies, sera from immunized mice, obtained 1 week and 10 weeks after the last booster injection, were tested for the presence of antibodies mediating complement-dependent lysis. Preimmune sera from the same mice were used as negative controls. As shown in Fig. 7, CaMBr1-CRM197 induced the production of antibodies mediating complement-dependent lysis of MCF7 cells. No reactivity was detected on A431 cells 57 days, or 120 days after vaccination in preabsorbed sera.

Discussion

In the present study, we report that the minimal antigenic structure within the Globo-H antigen can be optimally immunogenic in an animal model when properly presented by the carrier protein and administered in an appropriate vaccine formulation. This information provides the groundwork for the rational design of a

Table 1 Determination of antibody isotype produced after the vaccination with CRM197-tetrasaccharide plus MPL-SE. For each bleeding day, sera of two mice were pooled and binding activity was tested at different dilutions, in duplicate, by quantitative radioimmunoassay on tetrasaccharide linked to keyhole limpet hemocyanin. Relative amounts of Ig isotypes (relative ratio) are expressed as: [experimental radioactivity (cpm) detected using immune sera (1:2000) and anti-(mouse Ig subclass)-specific secondary antibody]/[background radioactivity (cpm)]. Background radioactivity (2744 cpm) was determined as the total binding of preimmune sera (diluted 1:125) detected by an anti-(mouse Ig) antibody directed against all isotypes. In parentheses is reported the reciprocal of serum titer. Titer was defined as the serum dilution at which binding levels three times higher than that of anti-(mouse Ig subclass)-specific secondary antibody alone were still detectable

Bleeding day	Relative ratio			
	IgM	IgG1	IgG2a	IgG2b
21	1.8 (2000)	4.7 (32 000)	2.2 (8000)	4.5 (16 000)
57	1.7 (2000)	7.9 (128 000)	7.9 (32 000)	8.9 (32 000)
120	1.5 (2000)	9.0 (64 000)	4.5 (16 000)	2.8 (8000)

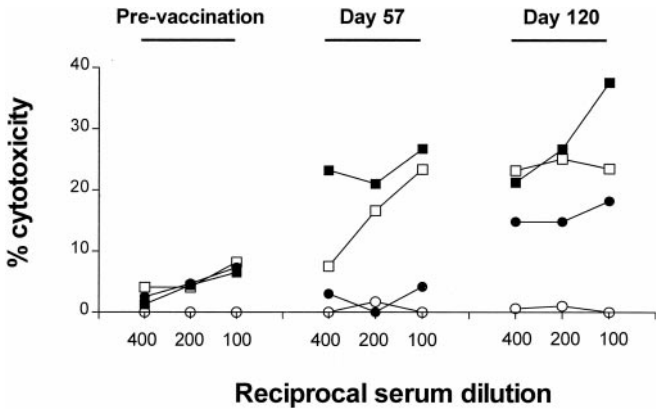

Fig. 7 Human tumor cell lysis by sera from vaccinated mice. Complement-dependent cytotoxicity of untreated (■, ●) or pre-absorbed (□, ○) sera against MCF7 and A431 cell lines was examined before vaccination and 57 days and 120 days after vaccination

highly specific reagent for active immunotherapy of CaMBr1-overexpressing carcinomas.

Most clinical trials using tumor cells or their extracts [10, 26, 35] as active specific immunization formulations have demonstrated only marginal effects, due mainly to the low reproducibility of the antigen preparation. Thus, new formulations have been developed, using synthetic or purified TAA. Saccharide antigens [29] appear to be good candidates for active specific immunization since they are largely expressed on the cell surface as components of gangliosides, glycolipids and glycoproteins [9], and a significant part of the humoral response of cancer patients is directed against them. Different glycoconjugates have been used for vaccination protocols in humans and there is evidence that natural and vaccine-induced antibodies correlate with improved disease-free and overall survival [21].

We previously proposed the synthetic tetrasaccharide epitope Fuc(α1-2)Galβ(1-3)GalNAc(β1-3)Galα-*O*-spacer [2] as the synthetic CaMBr1 antigen best-suited to be a vaccine against CaMBr1-overexpressing carcinomas. The tetrasaccharide preparation described here improved the synthetic yields and reduced the number of synthetic steps.

Saccharides are recognized mainly by T-cell-independent mechanisms and consequently there is no effective production of antibodies, especially those with high binding affinity. Furthermore, there is no induction of T cell memory required for booster responses. KLH appears to be the more potent carrier [13] and is already being used in a clinical anticancer vaccine formulation [34], whereas [14] CRM197 has already been approved for clinical vaccination trials with bacterial saccharides (from *Haemophilus influentiae* and *Neisseria meningitidis*) [7, 8, 30].

The adopted procedure for conjugation to KLH and CRM197, with the introduction of a long spacer between the carrier and saccharidic moiety, allows the conformation of the epitope to be preserved and probably results in its better presentation. In fact, we found that both conjugates induced an evident antibody response. The tetrasaccharide conjugated to KLH induced higher antibody titers than those elicited by CRM197. On the other hand, the KLH-induced response contained antibodies cross-reactive with other unrelated proteins, as demonstrated by absorption experiments. This might reflect the greater structural complexity and heterogeneity of KLH as compared to CRM197. Moreover CRM 197, but not KLH, induced a dose/response effect as expected for an effective vaccine formulation. Indeed, mice immunized with different doses of CRM197-tetrasaccharide exhibited a bell-shaped response curve with an optimal response at low–medium doses and a possible tolerogenic effect at higher doses.

Consistent with the results obtained with bacterial saccharides, CRM197 guides specific antibody production directed against the tumor-associated CaMBr1-saccharide moiety; after serum absorption on CaMBr1-negative cell lines, there was a significant increase in reactivity. By contrast, sera from mice immunized with KLH-CaMBr1 revealed a loss of binding capability.

In a variety of preclinical models, immunization with blood-group-associated antigens and gangliosides conjugated to KLH or BSA and administered with different adjuvants induced high-titer IgM and occasionally IgG antibody production [15, 29]. Furthermore, in a suitable syngenic mouse model, antibodies against the ganglioside GD2 were shown to eradicate circulating tumor cells and micrometastases [36], presumably by mechanisms activating complement-mediated lysis, antibody-dependent cell-mediated cytotoxicity and/or inflammatory reactions. In particular, when the entire Globo-H hexasaccharide was conjugated to KLH and administered with the adjuvant QS-21, it induced IgM and IgG responses (titers 1:128 000 and 1:2560 respectively) in immunized mice [29]. On this basis, a phase I dose-seeking and vaccine safety trial was conducted, which demonstrated that the Globo-H–KLH–QS-21 formulation was safe and able to induce specific high-titer IgM antibodies [34].

Our vaccine formulation generated a higher titer of IgG1 antibodies (titer 1:64 000) than the Globo-H–KLH–QS-21 formulation in mice, and the immune sera showed complement-mediated cytotoxic activity on CaMBr1-positive human tumor cells. These data suggest that the vaccine formulation, based on the synthetic minimal antigenic structure of Globo-H and on the use of a long saccharide-protein spacer and a simpler and more reproducible carrier (CRM197), might result in more efficient immune stimulation in humans.

Mice do not express CaMBr1 in normal tissues [12], so that the mouse immune system would be expected to recognize this antigen as foreign. In contrast, CaMBr1 immunization in humans may be hindered by a certain degree of tolerance. In this context, we recently demonstrated the usefulness of a rat model [3] for vaccines against human cancers over-expressing the CaMBr1

antigen. If the vaccine formulation identified in the present study (CaMBr1-CRM197 in association with MPL-SE, or possibly QS-21) proves to be effective in reducing rat liver implants of CaMBr1-positive rat colon carcinomas (unpublished results), it could represent a useful tool for adjuvant therapy in the human setting.

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