Expression profile of saccharide epitope CaMBr1 in normal and neoplastic tissue from dogs, cats, and rats: Implication for the development of human-derived cancer vaccines

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Summary

CaMBr1 is a blood group-related tumour-associated antigen, whose pattern of expression provides a therapeutic window for passive or active immunotherapy and points to the promise of a vaccine against carcinomas overexpressing this antigen. In this context, an animal model that closely mimics the human situation would be extremely useful. We, therefore, utilised the murine monoclonal antibody MBr1, which defines CaMBr1, as a useful probe to detect the molecule targeted for vaccine development on canine and feline spontaneous breast and uterus tumours and on their normal counterparts, and on rat normal tissues and carcinoma cell lines. Immunoperoxidase staining of cryostat sections revealed homogeneous CaMBr1 expression only in normal feline uterus and a uterus papilloma, whereas MBr1 reactivity was very weak and heterogeneous in normal (1/3 and 1/3) and tumour (1/10 and 1/6) breast tissues from dogs and cats, respectively. In contrast, the data obtained in rat tissues were reproducible in the strains tested and showed that CaMBr1 was expressed in all epithelial tissues of the digestive tract, although with variable intensities. Monoclonal antibody staining appeared to correspond to membrane-bound structures as well as mucinous secretions. Similarly, secretion products of lactating mammary glands expressed CaMBr1. The spectrum of expression on rat digestive tract was broader than that in humans but the specificity of MBr1 reactivity was confirmed by competition assay with a synthetic tetrasaccharide that mimics the CaMBr1 antigen. On FACS analysis, only one of two clonal derivatives of the rat breast carcinoma line RAMA 25 expressed CaMBr1, and a negative cell subset was evident in repeated experiments. By contrast, both colon carcinoma lines, DHD/K12 and CC531, showed staining with MBr1, albeit at different levels of intensity, and no evidence of a negative subset. The cell line CC531 maintained or even increased CaMBr1 expression levels following transplantation in syngeneic immunocompetent animals. Our data suggest the usefulness of the rat as a test model for vaccines against human cancers overexpressing the CaMBr1 antigen.

Introduction

The blood group-related tumour-associated antigen (TAA), CaMBr1, was first identified in the human breast carcinoma cell line MCF7 as identical to the globo-H antigen of terato-carcinoma using the murine monoclonal antibody MBr1 (Bremer *et al*. 1984). More recent studies have shown that the saccharidic epitope can be carried both on glycoproteins and glycolipids (Miotti *et al*. 1989), and that CaMBr1 overexpression is associated with poor prognosis in breast and small

cell lung carcinomas (Martignone *et al*. 1993, Perrone *et al*. 1993). We recently showed that the epitope consists of the tetrasaccharide Fucα1-2Galβ1-3GalNAcβ1-3Gal (Adobati *et al*. 1997), now available as a synthetic oligosaccharide (Lay *et al*. 1995, Toma *et al*. 1995). Like most other human TAA, CaMBr1 is also expressed on some normal tissues, namely mammary glands, uterine endocervical epithelium and glands, oviducts, pancreas acinar cells and ducts, distal and collecting tubules of the kidney (Mariani-Costantini *et al*. 1984a,b). However, the administration of up to 16 mg MBr1

to breast cancer patients caused no organ toxicity (Cascinelli *et al.* 1986). This might reflect the localisation of CaMBr1 on the luminal surface of kidney tubules and in the cytoplasm of pancreatic acinar cells. The expression of CaMBr1 in non-life-threatening tissues, the difference in antigen density between tumour and normal tissues, as well as the different cellular localisation, provide a therapeutic window for passive or active immunotherapy against cancer cells. These characteristics point to the promise of a vaccine against CaMBr1-overexpressing carcinomas.

Active specific cancer immunotherapy involves a wide range of approaches, all aimed at triggering the patient's immune system against neoplastic cells. Any strategy must be extensively studied in preclinical models and to date, most such studies have been performed in mice, undoubtedly the easiest and cheapest animal model. However, mice exhibit an expression pattern of glycosyltransferases different from that of humans (Hakomori 1996), so they do not express the majority of the described human saccharidic TAA, including CaMBr1 antigen. The mouse immune system is thus expected to recognise those TAA as foreign. In contrast, saccharidic TAA/CaMBr1 immunisation in humans may be hindered by a certain degree of tolerance and by potential targeting of normal tissues by TAA/CaMBr1 immune cells, leading to autoimmunity. In this context, an animal model that more closely mimics the human situation would be extremely useful. Indeed, some human TAA, including saccharidic ones, have been detected in tissues from different mammalian species, including monkeys, dogs, cats and rabbits (Clemo *et al.* 1995, Falini *et al.* 1989, Nouwen *et al.* 1990), and MBr1 was found to react with canine breast tissues (Mottolese *et al.* 1994). We therefore analysed CaMBr1 expression in canine and feline spontaneous breast tumours, and on rat normal tissues and syngeneic transplantable tumours. Our data suggest the usefulness of the rat as a test model for vaccines against human cancers overexpressing the CaMBr1 antigen.

Methods

Monoclonal antibodies and oligosaccharides

Murine MBr1 (IgM) was derived from Balb/c mice immunised with MCF7 human breast carcinoma cells as described (Ménard *et al.* 1983) and is directed to the terminal tetrasaccharide sequence of globo H (Adobati *et al.* 1997). It was purified by affinity chromatography on insolubilised anti-idiotype monoclonal antibody (MAb) A3B10 (Viale *et al.* 1987) as described previously (Adobati *et al.* 1997). Murine MAb B3 (IgG$_1$) was derived from Balb/c mice first tolerised by injection with normal human kidney membranes and cytoxan and then immunised with MCF7 cells (Pastan *et al.* 1991). MAb B3 reacts with Le^y antigen and was purified by affinity chromatography on insolubilised protein A.

MAb CC52, derived from mice immunised with rat colon carcinoma CC531 cells (Thomas *et al.* 1993), was used as a positive control in immunohistochemistry on liver heterotopic implants (see below).

Tetrasaccharide Fucα1-2Galβ1-3GalNAcβ1-3Galα-O-propyl and trisaccharide Fucα1-2Galα1-3GalNAcβ1-O-propyl were synthesised as described elsewhere (Lay *et al.* 1994, 1995) and dissolved in 0.15 M NaCl.

Normal tissues and tumours

Biopsies of feline and canine tissues were obtained from cats and dogs undergoing ovary-hysterectomy or mastectomy at the School of Veterinary Medicine, Milan University. The 6 mammary feline neoplasias were tubular adenocarcinomas. The 10 canine mammary tumours were more heterogeneous and classified according to the World Health Organisation guidelines for histological classification of neoplasms in domestic animals (World Health Organization 1974) as 4 tubular adenocarcinoma, 2 carcinoma, 1 mixed malignant tumour, 1 comedocarcinoma, 1 mastocytoma, and 1 complex tubular adenocarcinoma. Normal tissue specimens were collected from several rats from each of the following strains: the outbred strain Sprague–Dawley (CD) and the inbred strains Fisher 344, WAG/RIJ and BDIX. CD and Fisher 344 rats were purchased from Charles River (Calco, Italy). Wistar-derived WAG rats and BDIX rats were obtained from Harlan CPB (Zeist, The Netherlands) and from Iffa-Credo (L'Arbresle, France) respectively. Organs were collected at necropsy, immediately frozen in liquid nitrogen, and stored at $-80\,°$C.

Liver of ether-anaesthetised rats was exposed by laparotomy using a medial incision, and injected subcapsularly at 2 sites in both the left and right main lobes with 2.5×10^5 viable (Trypan blue exclusion) CC531 cells suspended in 50 µl buffered NaCl solution; 4 implants per liver were generated. Ten days after tumour cell injection, rats were sacrificed and laparotomy was performed; tumours were excised, snap-frozen in isopentane at $-70\,°$C and stored at $-80\,°$C.

Cell lines

Human breast cancer cell line MCF7 (from American Type Culture Collection, Rockville, MD, USA) was maintained in RPMI 1640 supplemented with 10% foetal calf serum (FCS). These cells overexpress CaMBr1 (Ménard *et al.* 1983) and Le^y (Pastan *et al.* 1991) epitopes.

Rat LA7 and 106AA10 cell lines (Dulbecco *et al.* 1979), a gift from Dr. I. Zucchi (CNR, Milan, Italy), were derived from a chemically induced breast carcinoma (RAMA25) in Sprague–Dawley CD rats and were grown in DMEM + 10% FCS + 55 ng/ml hydrocortisone + 50 ng/ml insulin. Rat CC531 (Marquet *et al.* 1984) and DHD/K12 (Martin *et al.* 1973) colon carcinoma cell lines, derived from WAG/RIJ and BDIX rats, respectively, were maintained in the same medium as MCF7 cells.

Rat fibroblast cell line RAT1 (Steimer & Klagsbrun 1981) was maintained in DMEM high glucose +5% FCS.

For each cell line, 10^5 cells were seeded on slide chambers and grown until semi-confluent or prepared as cytospin slides (900 rpm for 5 min), air-dried overnight, fixed in cold acetone for 10 min and stored at $-20\,^{\circ}$C.

Immunohistochemistry

Immunohistochemical analysis was performed on cryostat sections to avoid possible loss of the glycolipid fraction (Perrone *et al.* 1993). Sections from normal or neoplastic animal tissues, and cytospins and chamber slides from human and rat cell lines were air-dried overnight, fixed in cold acetone for 10 min, and analysed by immunoperoxidase staining using the biotin–peroxidase complex (ABC) method (Vector Laboratories, Burlingame, CA, USA) as described by Hsu *et al.* (1984). Briefly, sections were incubated in phosphate-buffered saline (PBS) + 1% H_2O_2 + 0.1% NaN_3 for 40 min to block endogenous peroxidase activity. After two washes in PBS, sections were saturated with RPMI + 5% FCS for 15 min and incubated with MAbs (10 µg/ml) for 60 min at room temperature. MAb binding was detected by sequential 30 min incubations with biotinylated anti-mouse rat IgG (1 : 100; Vector) adsorbed on rat tissue sections, or biotinylated anti-mouse IgM (1 : 50; Amersham) followed by ABC reagent with three rinses in PBS between incubations. Development was carried out by incubation with 3,3′-diaminobenzidine (Sigma) and H_2O_2 for 5 min. Sections were counterstained with haematoxylin. A MAb of irrelevant specificity was used as a negative control. Direct immunoperoxidase assay was performed on stomach, mammary gland and liver sections from rats, because these tissues reacted with ABC reagent. Acetone-fixed sections were treated in PBS + 1% H_2O_2 + 0.1% NaN_3 for 40 min to block endogenous peroxidases. After saturation with RPMI + 5% FCS for 30 min, sections were incubated for 60 min with MAb MBr1 or B3 (20 µg/ml) for stomach and mammary gland samples, or with MBr1 and CC52 culture supernatant (50 µg/ml and 1 : 10, respectively) for liver samples. After three rinses in PBS, binding was detected by incubation with peroxidase-conjugated rabbit anti-mouse Ig (Amersham) diluted 1 : 100 for 60 min. In the case of liver sections, a further incubation with peroxidase-conjugated swine anti-rabbit Ig (Dako, Denmark) was performed to increase the signal. The reaction was developed as described for the ABC method. Liver sections were not counterstained.

Competition between MAb MBr1 and oligosaccharides on tissue sections was assessed by incubating each section with a mixture of MBr1 (10 nM) and each oligosaccharide (100 and 10 µM) for 90 min at room temperature. Standard immunoperoxidase assay was carried out.

Reactivity on normal tissues was scored as: − negative, + faint, ++ positive and +++ strongly positive. Tumour sections and cell lines were scored as: − negative, + < 50% positive cells, ++ 50–90% positive cells or +++ > 90% positive cells.

Binding and competition assays on cultured cells

Cells were harvested by trypsin and washed twice with PBS + 0.03% bovine serum albumin (BSA) before testing. All assays were performed in ice; PBS + 0.03% BSA was used for washes and dilutions.

Each sample was incubated with 10 µg/ml MAb for 30 min. After two washes, MAb binding was detected with biotinylated anti-mouse IgM or IgG (1 : 50; Amersham) incubated for 30 min. Samples were washed twice and incubated with streptavidin-FITC (1 : 80; Amersham) for 30 min. Cell-bound fluorescence was determined by FACScan analysis (Becton-Dickinson). Competition assay on cell lines was performed as described previously (Adobati *et al.* 1997). Briefly, cells were incubated with a mixture of 1 nM MBr1 and serial dilutions of each oligosaccharide (100–1 µM) for 120 min on ice. After two washes, MBr1 binding was detected as described above.

Results

CaMBr1 expression in normal and tumour tissues from dog and cat

CaMBr1 was specifically expressed in 1/10 canine tumours and 1/3 normal canine breast with around 50% stained glands. Focal reactivity was found in 1/6 specimen of breast carcinomas from cat, while MAb staining was homogeneous in 1/3 feline normal breast. CaMBr1 was homogeneously positive in 7 specimens of normal feline uterus, both on endocervical and glandular epithelia, whereas its expression was significantly lower in 2 specimens, collected immediately after delivery, and detectable only on glandular epithelium. One uterus papilloma from cat expressed high levels of CaMBr1 (data not shown).

CaMBr1 expression on normal rat tissues

CaMBr1 was found on all epithelial tissues of the digestive tract, although with variable intensities. Since the stomach reacted with ABC reagent, a direct peroxidase assay was used to enable specific, although less intense, staining on the apical membranes of glandular epithelium; specific staining of stomach tissue was found in only 1 of 3 strains (Fisher 344) (Figure 1A). In all rat strains tested, MBr1 staining was most intense on the small (Figure 1C) and large intestine, especially on Brunner glands and along the surface of villi, where staining appeared to correspond not only to membrane-bound structures, but also to mucinous secretions (Figure 1D). No reactivity was observed in the stroma. Pancreas from WAG rats was CaMBr1-positive on Langerhans islets and ducts, while the other two strains were negative, perhaps due to poor conservation of the specimens. MAb B3, used as a positive control since Le^y is present on some rat tissues (Sprague–Dawley large intestine) (Trail *et al.* 1993), showed a weak reactivity on stomach only from the
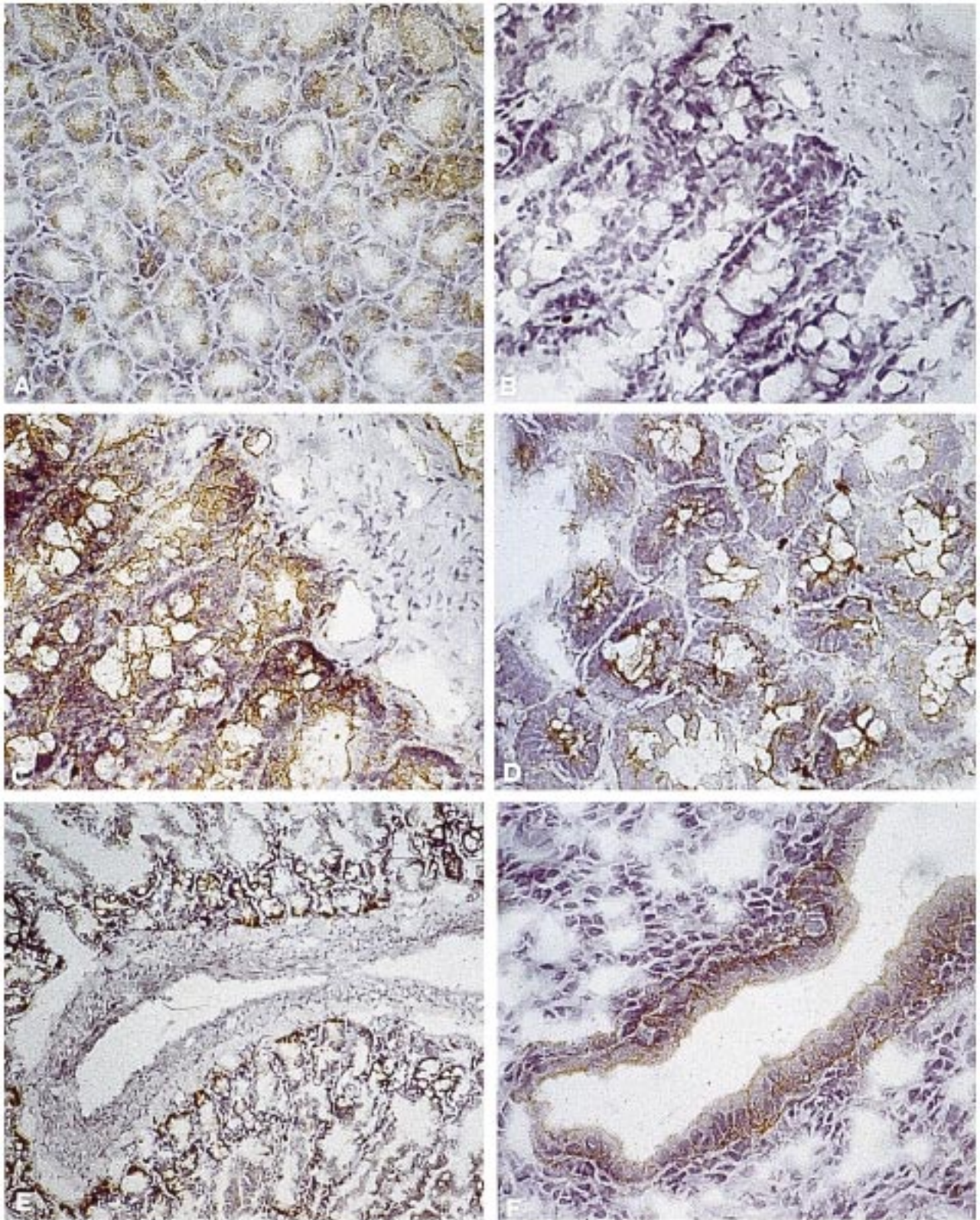
Figure 1. Immunohistochemical analysis of CaMBr1 and Le^y expression in normal rat tissues. Immunoperoxidase staining was carried out on 4–5 μm thick cryostat sections using the biotin–peroxidase complex (ABC) method on all tissues except stomach on which a direct peroxidase assay was performed. Diaminobenzidine was used as substrate, with Mayer's haematoxylin counterstain. Faint staining of apical membranes of glandular epithelium of the stomach was observed with MAb MBr1 only in the specimen from Fischer rat (A). In small intestine, no background staining with control unrelated MAb was observed (B). MAb MBr1 homogeneously stained the lining epithelium of small intestine specimen from a Lewis rat (C) and the Brunner glands and the surface of villi of large intestine specimen from a Fisher rat (D). Le^y epitope, as identified by MAb B3, was evident in large intestine epithelium from a Sprague–Dawley CD rat (E). MBr1 was strongly positive on uterine lining epithelium from the CD rat (F). No reactivity was observed in the stroma of any tissues tested. Magnification: ×25 (A,B,C,D,F); ×10 (E).

Fisher 344 rat. Le^y antigen was detected on villi and glands of small and large intestine of Sprague–Dawley rats (Figure 1E). The same distribution was observed in the other strains, although the staining intensity was weaker on sections from Fisher 344 rats.

Intense CaMBr1 expression was detected on uterine lining epithelium (Figure 1F) as well as on uterine glands, while oviducts and kidneys were negative. Focal reactivity of MAb B3 was detected in the kidney specimen from BDIX rats and on uterus from CD rats. Mammary epithelium of glands obtained from lactating CD female rats showed faint staining with both MAbs MBr1 and B3. Table 1 summarises these data with published human tissue reactivity included for comparison (Ménard *et al.* 1983, Mariani-Costantini *et al.* 1984a,b, Perrone *et al.* 1990, Pastan *et al.* 1991).

To assess the specificity of MAb MBr1 binding, a competition assay was performed on each positive tissue with two synthetic oligosaccharides: a tetrasaccharide that mimicks CaMBr1 (Adobati *et al.* 1997) and a related trisaccharide, which lacks the internal galactose residue and shows an anomeric structure in the Gal–GalNAc linkage (see Methods for structures). The synthetic epitope inhibited the staining in a concentration-dependent manner, with complete absence of staining at 100 μM oligosaccharide; this pattern of inhibition closely resembles that observed on MCF7 cells (Adobati *et al.* 1997). At the same concentration, the trisaccharide did not modify the staining intensity.

Expression of CaMBr1 on rat tumour cell lines

Four rat carcinoma cell lines were analysed for expression of CaMBr1. The rat fibroblast cell line RAT1 and the human breast carcinoma cell line MCF7 were used as negative and positive controls, respectively (Figure 2). As expected, MAb MBr1 strongly reacted with MCF7 human cells (mean fluorescence intensity, 493) and was completely negative with RAT1 cells. Only one of the two clonal derivatives of rat breast carcinoma RAMA25 cells (106AA10) was positive for MAb MBr1 binding, and repeated experiments confirmed the bimodal distribution with a negative cell subset and 50–70% of cells with heterogeneous labelling. Colon carcinoma cell lines DHD/K12 and CC531 expressed CaMBr1 at low and medium levels respectively. Heterogeneous staining was confirmed in repeated experiments, but no clear bimodal distribution was observed. In all experiments, DHD/K12 cells expressed the lowest level of CaMBr1 (mean fluorescence intensity, 91) as compared to the other two positive rat cell lines (mean fluorescence intensity for 106AA10 and CC531, 430 and 158, respectively). The anti-Le^y MAb B3 showed specific and intense reactivity only with control MCF7 human cells whereas all rat cancer cells were completely negative (data not shown).

Competition assay of MBr1 binding to the three positive rat cell lines, as well as to the positive control cell line MCF7, revealed inhibition by the tetrasaccharide but no effect using

Table 1. Expression of CaMBr1 and Le^y on rat normal tissues.[a]

	CaMBr1		Le^y	
	Rat	Human[b]	Rat	Human/primate
Digestive tract				
Stomach	1/3(+)[c]	No	1/3(+)[c]	Yes Glands, mucin
Small intestine	4/4(+++)	Yes Brunner glands	3/3(+/+++)	Yes Mucin
Large intestine	4/4(+++)	No	3/3(+/+++)	No/yes Epithelium
Pancreas	1/3(++)[d]	Yes Acini	0/3	Yes Acini and ducts
Urogenital system				
Kidney	0/3	Yes Distal tubules	1/3(+)[e]	No
Oviducts	0/2	No	0/2	No
Uterus	2/2(+++)	Yes Endocervix	1/2(+)[f]	Yes[g]

[a]By immunoperoxidase staining of specimens from 4 rat strains. Data are given as number of positive strains, with intensity of staining scored as: − negative, + faint, ++ positive, +++ strongly positive in parentheses.
[b]Detailed data for human tissue reactivity are given in Refs. (Ménard *et al.* 1983, Mariani-Costantini *et al.* 1984a,b, Perrone *et al.* 1990) for Mab MBr1 and Ref. (Pastan *et al.* 1991) for Mab B3.
[c]Positive specimens: Fisher 344.
[d]Positive specimens: WAG .
[e]Positive specimens: BDIX.
[f]Positive specimens: CD.
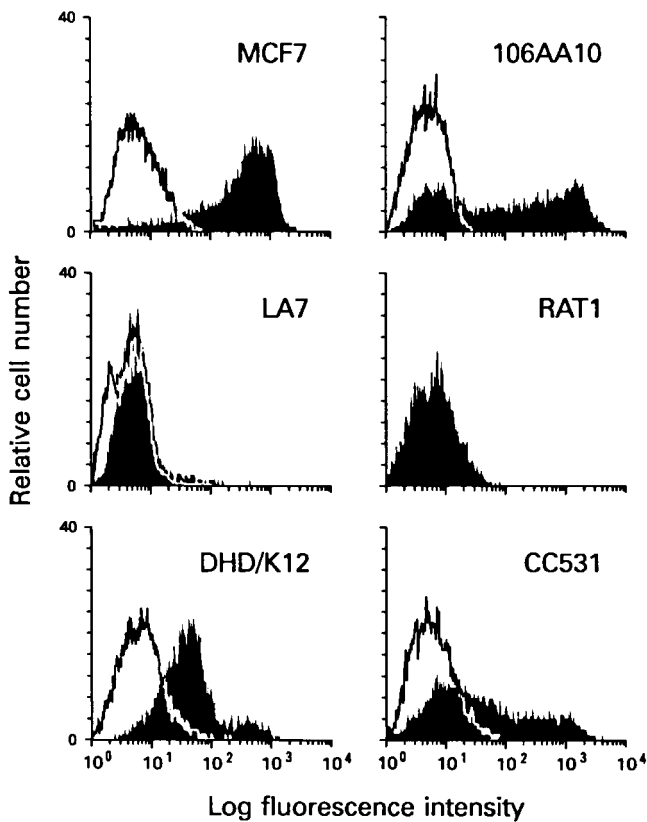[g]Cynomologus monkey.

Figure 2. FACS analysis of MAb MBr1 binding the following rat cell lines: 106AA10 and LA7 (breast carcinoma from Sprague–Dawley CD rat), RAT1 (normal fibroblast from CD rat), DHD/K12 and CC531 (colon adenocarcinoma from BDIX and WAG rats respectively). Human breast carcinoma cell line MCF7 was used as a positive control. Cells were incubated in ice with MBr1 MAb at 10 μg/ml for 30 min. Binding was detected by incubation with biotinylated anti-mouse IgM and IgG antibodies followed by FITC-conjugated streptavidin. Open histograms: cells reacted with secondary antibody and FITC-conjugated streptavidin only. Filled histograms: cell reacted with MAb MBr1.

the trisaccharide. The immunofluorescence data were consistent with those obtained in repeated immunohistochemistry assays on cytospins and on adherent cells, grown in chamber slides and acetone-fixed. While MCF7 cells stained homogeneously with MBr1, the three rat cancer cell lines showed areas of CaMBr1-positive cells and patches of negativity that were particularly evident in 106AA10 cells; in each case, staining was at the membrane level, and no morphological difference between positive and negative cells was detectable.

The two colon carcinoma lines were obtained in a syngeneic strain and therefore could be maintained as *in vivo* transplants (Marquet *et al*. 1984, Martin *et al*. 1973). These cell lines, when injected directly in the liver, grew locally, mimicking colon metastatic growth. Analysis of MBr1 reactivity on heterotopic implants of CC531 cells in the liver of WAG rats revealed membrane staining in the majority of cancer cells infiltrating the liver (Figure 3A), although staining was less homogeneous and less intense than that observed with the positive control MAb CC52 (Figure 3B).

Discussion

Our conceptual approach to active immunotherapy of CaMBr1-overexpressing tumours is the development of a carbohydrate vaccine consisting of the synthetic tetrasaccharidic epitope conjugated to an appropriate protein carrier. The present data identify the rat as a potentially useful system in which to test simultaneously the efficacy of such a vaccine in tumour cell growth and the possible detrimental effects on normal tissue.

Based on a previous study on canine tumours (Mottolese *et al*. 1994) in which MAb MBr1 was found to react with 87% of canine normal breast tissue and benign mammary tumours and with 93% of canine mammary carcinomas, we evaluated spontaneous feline and canine tumours. However, we found great heterogeneity in expression among matched tissue types, and the level of staining was much lower than expected. Our results raise the possibility that MBr1 expression might depend on the tumour histotype and on the hormonal status of the animal at the time of biopsy, but clearly indicate the unsuitability of this animal model. Unlike the previous analysis in which MAb MBr1 was left to react at 4 °C overnight at high concentrations (25–50 μg/ml) with histological sections, our data were obtained using MBr1 at 10 μg/ml for 1 h at room temperature on cryostat sections. We, therefore, speculate that the discrepant results reflect the very low level of expression of the saccharide epitope in these animals. Moreover, MAb in the previous study was purified by gel filtration and not by affinity chromatography, and no competition assays were done to confirm specificity; thus, cross-reactivity of the MAb on canine sections with similar saccharidic antigens cannot be excluded.

In contrast, the data obtained in rat were reproducible and showed that the CaMBr1 expression pattern was consistent with that in humans (Ménard *et al*. 1983, Mariani-Costantini *et al*. 1984a,b, Perrone *et al*. 1990), i.e., expression in some normal tissues, generally polarised on the luminal surface, and a quite homogeneous expression in at least a transplantable tumour cell line. We focused our analysis on digestive tract tissues because their physiologies are more similar among mammals than are, for example, reproductive tissues. A similar tissue distribution was observed for both CaMBr1 and Le^y epitopes in rat and human normal specimens although rat large intestine showed stronger staining than the human tissue. For both antigens, MAbs staining appeared to correspond to membrane-bound structures as well as mucinous secretions. Similarly, secretion products of lactating mammary glands expressed Ca-MBr1. Since the spectrum of reactivity on rat digestive tract tissues was broader than that in humans, we evaluated the specificity of MBr1 reactivity by competition assay with the synthetic epitope. The pattern of inhibition, which closely resembled that observed on human cells (Adobati *et al*. 1997), confirmed the staining data. Mammals display a unique pattern of distribution of the α-1,3-galactosyltransferase, according to which all non-primate mammals synthesise and express on glycoconjugates an abundance of α-galactosyl epitope (Galili 1993) while
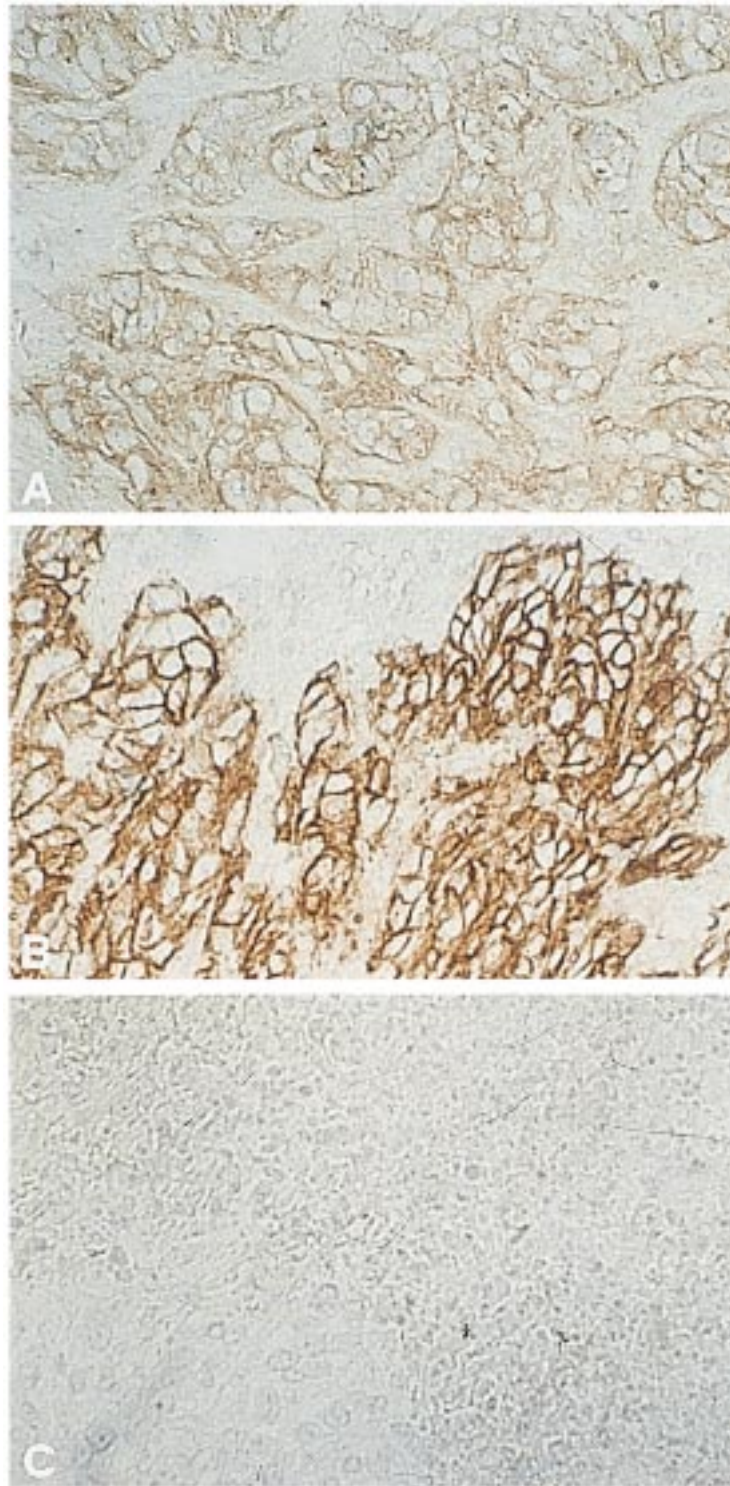
Figure 3. Immunohistochemical analysis of CaMBr1 expression on heterotopic implant of the rat colon carcinoma CC531 in liver of a WAG rat. A direct peroxidase assay was performed on 4–5 μm cryostat sections of WAG liver collected 10 days after tumour cell injection. To better visualise the pale brown labelling of diaminobenzidine chromogenic substrate, no counterstaining was applied. Both MBr1 (A) and CC52 (B) MAbs stained the colon carcinoma cells, but the reactivity of MBr1 was less homogeneous and intense. No background staining was observed either in normal liver or in infiltrating colon carcinoma cells in absence of MAb (C). Magnification ×25.

catharrhines (Old World monkeys, apes and humans), which lack the enzyme, are totally devoid of this epitope (Galili & Swanson 1991). Apart from this difference in glycosyltransferases, our present data, together with the earlier biochemical characterisation of glycosphingolipids of rat gastrointestinal tract (Breimer *et al.* 1982) strongly support the hypothesis that the pattern of terminal glycosylation in rat and humans is very similar.

Only one of the two clonal derivatives of the rat breast carcinoma line expressed CaMBr1 and a negative cell subset was evident in repeated experiments. In this case, antigen expression may be dependent on hormonal regulation, as observed in women (Perrone *et al.* 1990), making it difficult to maintain the proper conditions to use this line as a preclinical model. Moreover, this cell line was derived in an outbred strain (Dulbecco *et al.* 1979) and consequently cannot be transplanted in immunocompetent animals. In contrast, both colon carcinoma cell lines examined showed staining with MAb MBr1, albeit at different levels of intensity, and no evidence of a negative cell subset. Unlike human colon carcinoma cells (Pastan *et al.* 1991), the rat counterpart did not express Le^y antigen and at present we do not have an explanation for this discrepancy.

The colon carcinoma cell line CC531 maintained or even increased CaMBr1 expression levels following transplantation in syngeneic immunocompetent animals. The non-homogeneous staining is consistent with that observed in human specimens (Ménard *et al.* 1983, Mariani-Costantini *et al.* 1984a,b, Martignone *et al.* 1993, Perrone *et al.* 1993). In the context of obtaining a suitable animal model for immunotherapy against CaMBr1-expressing carcinomas, the presence of a tumour cell subset with undetectable amounts of target antigen should not be regarded as a major drawback. Heterogeneity in antigen expression is also a well-known characteristic of human tumours (Greiner *et al.* 1987); thus, the efficacy of any immunotherapeutic approach is likely to rely not only on a direct cytotoxic activity against cells expressing the target antigen, but also on a 'bystander effect' (Colombo & Forni 1997).

Since the luminal surface of secretory cells is largely inaccessible to the immune system, the induction of an immune response against CaMBr1 is not expected to lead to autoimmunity against essential organs. Confirmation of this and of the anti-tumour efficacy against the liver implant of the rat colon carcinoma CC531 cells awaits actual testing of the vaccine presently under development.

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