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Anti-GM_3-lactam monoclonal antibodies of the IgG type recognize natural GM_3-ganglioside lactone but not GM_3-ganglioside

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Immunization of mice with a synthetic GM_3-lactam-BSA (bovine serum albumin) conjugate (designed to emulate the corresponding natural GM_3-lactone conjugate), followed by fusion of splenocytes with myeloma cells, gave rise to more than 300 monoclonal hybridomas producing antibodies to GM_3-lactam-BSA, which did not react with Glc-BSA and BSA. Eight antibody clones were randomly chosen from the positive 300 hybridomas. The eight clones, all belonging to the IgG class, were unreactive against GM_3-ganglioside, whereas two antibodies (P5-1 and P5-3, both IgG_1, κ) reacted with GM_3-ganglioside lactone. Binding of these two antibodies to the GM_3-lactam-BSA conjugate was inhibited by soluble glycosides of GM_2-, GM_3-, and GM_4-lactam and by GM_3- and GM_4-lactam, respectively, but not by Gb_3 or asialo-GM_1 and GM_2-saccharides. A third antibody (P3; IgG_{2b}, κ) was inhibited by GM_2-, GM_3-, and GM_4-lactam, but did not recognize GM_3-ganglioside lactone.

Keywords: anti-GM_3-lactam and anti-GM_3-lactone monoclonal IgG antibodies

Hakomori and coworkers [1, 2] have established two monoclonal antibodies by immunization with syngeneic B16 melanoma cells and with GM_3-ganglioside lactone (**3**) coated on *Salmonella minnesotae*. IgM and IgG antibodies were obtained, each recognizing both GM_3-ganglioside (**1**) and GM_3-ganglioside lactone (**3**). Their reactivity depended on how the hapten was presented to the antibody. Recently, an antibody was reported that specifically recognized disialoganglioside lactone and not the open form of the ganglioside [3]. GM_3-Ganglioside lactone was found to be much more immunogenic than GM_3-ganglioside, and it was suggested that ganglioside lactones could be useful immunogens for active immunization against tumours.

Ganglioside lactones are formed upon acidification of gangliosides, but are rapidly hydrolysed at neutral or basic pH. Therefore, it is not likely that a high lactone concentration can be maintained *in vivo*. We devised a synthetic route to GM_3-ganglioside *lactam*, including its BSA conjugate (**4**), suitable for immunizations. Ganglioside lactams should be hydrolytically stable at physiological pH while still being conformationally similar to the corresponding lactones [4].

In the present paper, we describe the immunization of Balb/c mice with GM_3-lactam-BSA (**4**), and the establishment of several hybridomas, secreting IgG antibodies, two of which recognized both GM_3-lactam and GM_3-lactone, but not the open (nonlactonized) form of GM_3-ganglioside. The specificities of three of the antibodies (P3, P5-1, and P5-3) were investigated in greater detail, as shown below. The antibodies might find use in, for example, an immunohistological search for GM_3-ganglioside lactone (**3**) in various tissue preparations.

GM_3-lactam-BSA seems to be the first example of a designed, synthetic, non-natural antigen giving rise to antibodies that cross-react efficiently with the natural hapten. Furthermore, ganglioside lactams are made up of natural monosaccharides and might therefore be natural products yet to be discovered.

Materials and methods

Establishment of monoclonal antibodies

The method of Köhler was followed [5]. The GM_3-lactam-BSA conjugate [4] (**4**, 50 μg) was dissolved in Dulbecco phosphate-buffered saline (PBS, pH 7.2, 500 μl), mixed with

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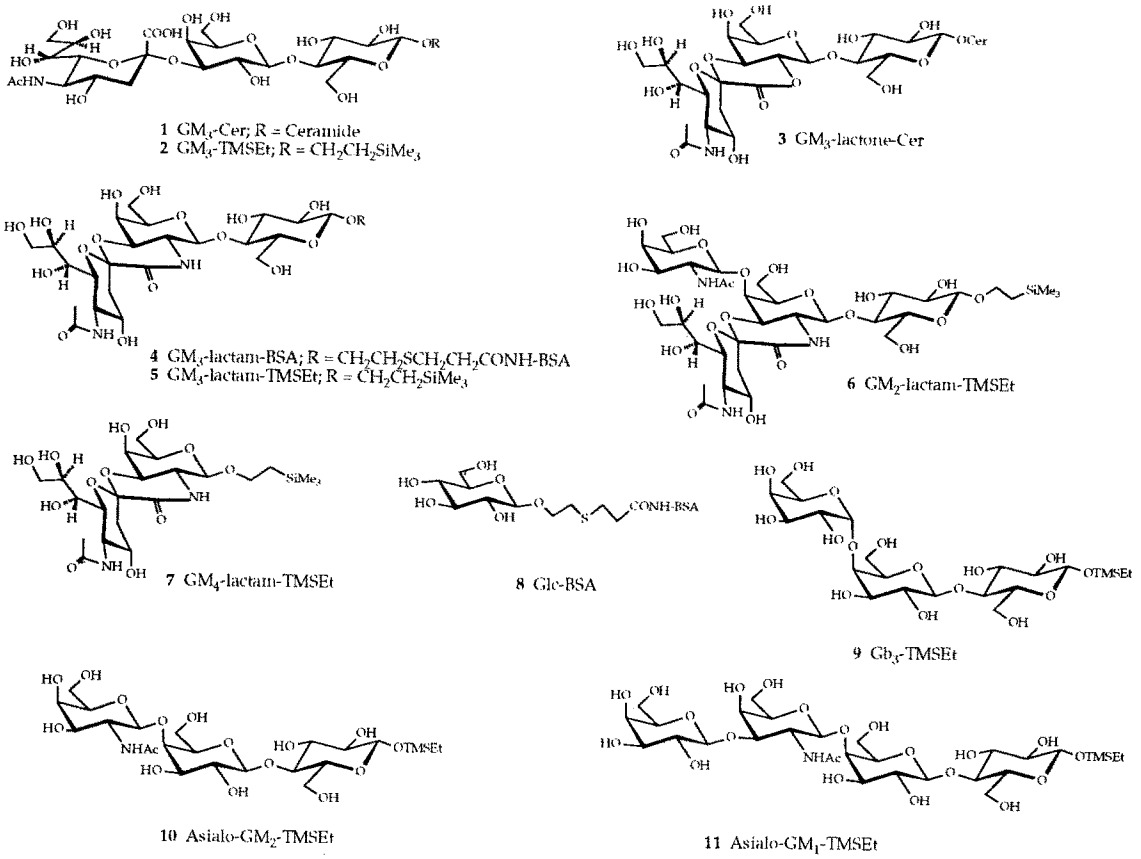

Figure 1. Chemical structures of the various substances used for immunization and specificity testing of antibodies.

500 µl Freund's complete adjuvant (FCA, Sigma, St. Louis, USA) and injected subcutaneously (s.c.) into a Balb/c mouse. The immunization, now using the antigen with Freund's incomplete adjuvant (FIA), was repeated three times (s.c.) with one week intervals. After 20 days, the mouse received a final intravenous (i.v.) booster dose (50 µg **4** in 100 µl of PBS) and seven days later it was splenectomized. The spleen cells were fused with Sp2/0 myeloma cells at the ratio 1:4 and hybridomas were screened for reactivity with GM_3-lactam-BSA coated plates (**4**, 3 µg ml^{-1}) using ELISA (see below). A synthetic glucose-containing BSA conjugate (**8**, prepared by the same route [4] as **4**), corresponding to the inner part of **4**, and BSA were used as negative controls. Cells from positive wells were expanded and recloned. More than 300 monoclonal hybridomas were established. Eight of the hybridomas were chosen at random and their specificities were investigated.

Tissue culture

Hybridoma cell lines were maintained in RPMI 1640 medium supplemented with 5% fetal calf serum, 100 IU ml^{-1} penicillin and 1:50 dilution of hypoxanthin–thymidine (H.T.) supplement. All tissue culture media and supplements were from Gibco Ltd, Scotland.

Specificity testing by binding to neoglycoproteins and glycolipids (Table 1)

GM_3-lactam-BSA (**4**), Glc-BSA (**8**), BSA, GM_3-ganglioside (**1**; obtained from BioCarb Chemicals, Sweden) and GM_3-ganglioside lactone (**3**) [6] were used for coating of microtitre plates using the method described by Nores *et al.* [1]. All the conjugates and compounds were used at concentrations of 3 µg ml^{-1}. Antibodies (100 µl supernatant) were added to each of the coated wells and the amount of bound antibody was detected using ELISA as described below.

ELISA screening

ELISA was performed by first binding the neoglycoproteins **4** and **8**, and BSA (3 µg m^{-1} in 50 mM $NaHCO_3$ buffer, pH 9.6, 100 µl), and the gangliosides **1** and **3** (6 µg ml^{-1} in CH_3OH, 50 µl) to ELISA microplates (Costar, USA). The plates were left overnight at 21 °C in a humidified atmosphere (neoglycoprotein plates), or in a ventilated hood (gangliosides) and then blocked with a BSA solution (1% in PBS buffer, 200 µl per well) for 30 min. The plates were washed with 3 × 200 µl washing buffer (PBS plus 0.05% Tween 20). Hybridoma supernatant (100 µl) was added to each well,

Table 1. Binding specificity and subclass of eight randomly chosen monoclonal antibodies obtained by immunization with GM_3-lactam-BSA (**4**).[a]

Antibody	*Saccharide used for coating*				*BSA*	*Subclass*
	4	**3**	**1**	**8**		
P2-D10-H4-H9 (P2-1)	+++	–	–	–	–	IgG_{2b}, κ
P2-E12-C9-F9 (P2-2)	+++	–	–	–	–	IgG_{2b}, κ
P3-A12-H4-F3 (P3)	+++	–	–	–	–	IgG_{2b}, κ
P4-C7-H9 (P4-1)	+++	–	–	–	–	IgG_{2b}, κ
P4-C7-G10-A6 (P4-2)	+++	–	–	–	–	IgG_{2b}, κ
P5-F12-D8-B2 (P5-1)	+++	++	–	–	–	IgG_1, κ
P5-F12-E4-D4 (P5-2)	+++	–	–	–	–	IgG_1, κ
P5-F12-G5-E6 (P5-3)	+++	++	–	–	–	IgG_1, κ

[a] +++, Strong binding, ELISA optical density reading >1.5 at 405 nm; ++, moderate binding, ELISA optical density reading ~1 at 405 nm; –, no binding.

the plates were incubated for 2 h at 21 °C, and washed with 3 × 200 µl washing buffer. Rabbit anti-mouse-Ig/alkaline phosphatase-conjugate (Dako A/S, Denmark) in PBS containing 0.1% BSA was added to each well, and the plates were incubated for 1 h at 21 °C, and washed as above. The phosphatase substrate (*p*-nitrophenyl phosphate, 5 mg, tablet, Sigma Diagnostics, USA) was dissolved in 10 ml substrate buffer (pH 9.8, 97 ml diethanolamine and 101 mg $MgCl_2 \cdot 6H_2O$, dissolved in H_2O to a volume of 1000 ml) and added to the wells (200 µl per well). The plate were incubated for 30 min at 37 °C and the optical density (OD) was measured at 405 nm with a Titertek Multiscan photometer (Flow Labs Ltd, Scotland). Sera from immunized and pre-immunized Balb/c mice were used as positive and negative controls, respectively.

Specificity testing by inhibition (Fig. 2)

The following 2-(trimethylsilyl)ethyl glycosides (Fig. 1) were synthesized and used in the inhibition studies. The compounds were pure according to TLC and NMR. GM_3-TMSEt[a] (**2**), GM_3-lactam-TMSEt (**5**) [4], GM_2-lactam-TMSEt {**6**, synthesized by glycosylation of a derivative of **5**, having HO-4′ unprotected, and removal of protecting groups. Compound **6** had $[\alpha]_D^{22} - 29°$ (*c* 0.8, MeOH). ^{1}H-NMR (^2H_2O) δ 4.68 (d, 1H, *J* 8 Hz, H-1′), 4.62 (d, 1 H, *J* 8 Hz, H-1‴), δ 4.47 (d, 1 H, *J* 8 Hz, H-1)}, GM_4-lactam-TMSEt {**7**, synthesized by sialylation of a derivative of galactosamine essentially as described in the synthesis of **5** [4]. Compound **7** had $[\alpha]_D^{25} - 13°$ (*c* 1, H_2O). ^{1}H-NMR (^2H_2O) δ 4.57 (d, 1 H, *J* 8 Hz, H-1)}, Gb_3-TMSEt (**9**) [7], asialo-GM_2-TMSEt (**10**) [8], and asialo-GM_1-TMSEt (**11**) [8].

Each of the glycosides was dissolved in PBS buffer with 0.1% BSA to give a 2 mM solution, which was sequentially diluted with 5 volumes of PBS buffer in glass tubes. An aliquot (160 µl) of each saccharide solution was added to aliquots of the antibody solution (160 µl supernatant), the plates were incubated overnight at 4 °C, and tested by ELISA (Fig. 2).

Isotyping of monoclonal antibodies

Ig class and subclass testing of hybridomas was performed with a commercial dip-stick kit (Holland Biotechnology, The Netherlands).

Results and discussion

By immunization of mice with GM_3-lactam-BSA (**4**), followed by establishment of hybridomas, we found a large number (>300) of antigen-specific clones. The majority of these clones recognized the sialyl-lactam-galactose portion of the antigen (see below); the remaining clones recognizing Glc-BSA (**8**) or BSA were not processed further. Considering the low immunogenicity of gangliosides in general, it is of special interest to note the high IgG response to GM_3-lactam, indiating its highly 'non-self' structure and immunogenicity.

Of the large number of sialyl-lactam-galactose specific clones, eight hybridomas, chosen at random, produced antibodies that were found to belong to the IgG, κ class (Table 1). They all bound to the antigen (**4**) attached to microtitre plates but did not bind to BSA, Glc-BSA (**8**), or GM_3-ganglioside (**1**). Two of the antibodies (P5-1 and P5-3) reacted also with GM_3-ganglioside lactone (**3**), thus meeting our objective to use a saccharide analogue (**4**) for immunization in order to obtain antibodies recognizing its natural counterpart (**3**). It should be noted that some of the remaining antibodies might possess binding characteristics similar to or different from the ones investigated here.

Binding of antibodies P5-1 and P5-3 to **4** adsorbed on microtitre plates was inhibited by soluble **4** and by the lactams **5** and **7**, whereas the glycosides **2**, **6**, **9**, **10**, and **11** were inefficient as inhibitors (Fig. 2). The fact that GM_2-lactam-TMSEt (**6**) was inactive indicates that the antibodies recognize the epitope of lactamized (and lactonized) GM_3-saccharides, that in the GM_2-lactam (**6**) carries a sterically hindering *N*-acetylgalactosamine residue. Binding of antibody P3 (which did not recognize GM_3-ganglioside lactone **3**) to compound **4** was inhibited by the lactams **5**, **6**, and 7. Obviously, the *N*-acetylgalactosamine residue of **6** did not hinder the binding. Thus, antibodies P3 and P5-1/P5-3 seem to recognize different saccharide epitopes. Since antibodies P5-1 and P5-3 recognize GM_3-ganglioside lactone (**3**) but not the open form of GM_3-ganglioside (**1**) (Table 1), they are potentially useful for selective immunohistological detection of GM_3-ganglioside lactone (**3**).

Of principal importance is the fact that these antibodies recognize both the natural GM_3-lactone *and* the synthetic

[a] Gift from Dr J. Dahmén, Symbicom AB, Ideon Research Park, Lund, Sweden.

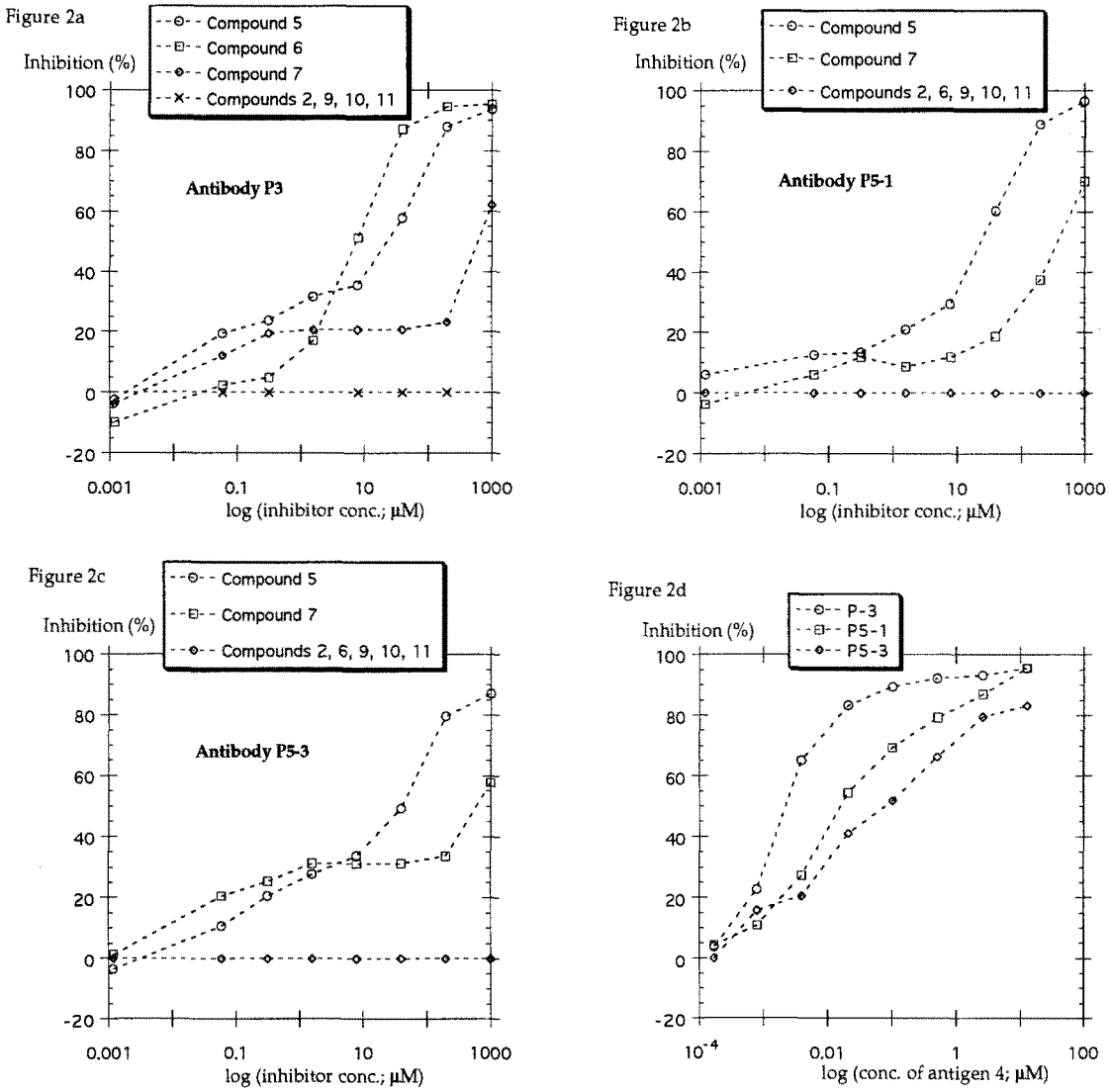

Figure 2. Inhibition of the binding of monoclonal antibodies P3, P5-1 and P5-3 to GM_3-lactam-BSA (**4**), using the soluble inhibitors **2**, **5–7**, **9–11**: (a) inhibition of P3; (b) inhibition of P5-1; (c) inhibition of P5-3; (d) inhibition of P3, P5-1, and P5-3 with GM_3-lactam-BSA (**4**).

GM_3-lactam, which means that the saccharide conformations are very similar when bound by the antibodies. (The similarity of the solution conformations has already been demonstrated [4].) Therefore, stable ganglioside lactams might be useful substitutes in general for unstable ganglioside lactones in active immunization against ganglioside-expressing tumours and in other biomedical investigations.

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