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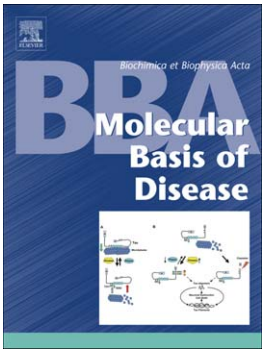

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EXPERIMENTAL GUILLAIN-BARRE SYNDROME INDUCED BY IMMUNIZATION WITH GANGLIOSIDES: KEYHOLE LIMPET HEMOCYANIN IS REQUIRED FOR DISEASE TRIGGERING.

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Abbreviated title: KLH in experimental Guillain-Barré syndrome.

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Abbreviations

GA1: $GgOse_4Cer$;

GM1: $II^3NeuAcGgOse_4Cer$;

GD1a: $IV^3NeuAcII^3NeuAcGgOse_4Cer$;

GD1b: $II^3(NeuAc)_2GgOse_4Cer$;

GT1b: $IV^3NeuAcII^3(NeuAc)_2GgOse_4Cer$

Abstract

An experimental model of Guillain-Barré Syndrome has been established in recent years. Rabbits develop disease upon immunization with a single dose of an emulsion containing bovine brain gangliosides, KLH and complete Freund's adjuvant. Within a period of four to ten weeks after immunization, they began to produce anti-ganglioside IgG-antibodies first, and to show clinical signs of neuropathy afterwards. In addition to gangliosides, KLH is a requirement for antibody production and disease triggering. Although KLH is commonly used as an immunological carrier protein, an anti-KLH-specific immune response was necessary for induction of both events. KLH is a glycoprotein carrying most of the immunogenicity in its glycan moiety. Between 20% to 80% of anti-ganglioside IgG-antibodies present in sick rabbit sera cross-reacted with KLH, indicating that both immune responses are related. The terminal Gal-ß(1,3)-GalNAc glycan (present in gangliosides and KLH) is proposed as "key" antigenic determinant involved in inducing the anti-ganglioside immune response. These results are discussed in the context of the "binding site drift" hypothesis.

Key words: Anti-ganglioside antibodies, KLH, Guillain-Barré syndrome, experimental model, binding site drift.

1. Introduction

Guillain-Barré syndrome (GBS) is an autoimmune neuropathy with a high mortality rate [1]. Gangliosides (self glycan-carrying molecules enriched in neural tissues) have been proposed as the main antigenic targets for antibodies mediating this disabling process [1-3]. Although intravenous immunoglobulin and plasma exchange have been successful therapeutic tools, current research is focused on the immune mechanisms involved in triggering the disease, aiming for an early immune intervention. Since Nagai et al. [4] described that ganglioside immunization induce a motor neuropathy (so called “ganglioside syndrome”), several laboratories have searched for an animal model of the disease with controversial results. Rabbits inoculated with GM1 in Freund's adjuvant produced anti-GM1 antibodies, but showed only minor electrophysiological and pathological changes [5] with no clinical symptoms [6]. In 2001, Yuki´s laboratory [7] described an experimental model of GBS by immunizing rabbits with bovine brain gangliosides (BBG). Yuki´s immunization protocol was slightly different than those previously used by other laboratories. Unlike earlier protocols using bovine serum albumin (BSA), it included keyhole limpet hemocyanin (KLH) as carrier protein in the immunogen. Although our laboratory also failed to produce the “ganglioside syndrome” in a 2002 experiment [6], we were able to reproduce Yuki´s model of GBS more recently using KLH [8]. Herein we characterize the critical role played by KLH in antibody induction and disease triggering.

2. Materials and methods

2.1. Gangliosides

Gangliosides GM1, GD1a, GD1b, and GT1b were prepared from human brain and used as antigens. Folch upper phase of lipid extract [9] was purified by reversed phase chromatography [10] and DEAE-Sephadex column chromatography [11]. Mono-, di-, and tri-sialoganglioside fractions were obtained by sequential elution from the column, using 10, 25, and 40 mM sodium acetate in methanol, respectively. GM1, GD1a, GD1b, and GT1b were purified from their proper fractions by HPLC, using an Iatrobead 8010 silica column and mixtures of isopropanol/hexane/water as running solvent [12]. Asialo-GM1 (GA1) was prepared by acid hydrolysis of bovine brain gangliosides [13], and further purified by DEAE-Sephadex and HPLC. Total ganglioside fraction of bovine brain (BBG) was prepared by Folch extraction, DEAE-Sephadex, alkaline methanolysis, and reversed phase chromatography. This preparation of BBG was composed by 4 major gangliosides: GM1 (30 %), GD1a (48 %), GD1b (10 %) and GT1b (12 %). The preparation did not contain GA1.

2.2. Immunization of rabbits

Five hundred microliters PBS containing 2.5 mg BBG and 1 mg KLH (Calbiochem, San Diego, CA) or methylated BSA (Sigma, St. Louis, MO, USA) were emulsified in 500 μl complete Freund's adjuvant (CFA). Emulsification was accomplished by repetitive passing mixture throw a needle (0.5 x 40 mm) using a disposable syringe. New Zealand male white rabbits weighting 2.5 -3 kg were injected subcutaneously on the back (5 x 0.2 ml). Control animals were

immunized with the same inoculum without BSA, KLH or ganglioside, under the same protocol. Rabbits were weighed and revised weekly to detect appearance of clinical signs. Blood samples were taken by ear vein puncture; sera were separated from blood clots and frozen at -70°C until use. Several experiments with a total of 27 rabbits were performed, all in accordance with international and institutional guidelines for animal care.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Fifty picomoles GM1 in 50 μl methanol were pipetted into microtiter plate wells, and dried overnight at 37°C. Alternatively, 50 μl of a solution of KLH (2μg/ml) in sodium carbonate buffer, 0.1 M, pH: 8.3 were incubated at 4°C overnight and washed. Each well was blocked with 1% BSA (Sigma, St. Louis, MO, USA) in phosphate buffered saline (BSA-PBS) for 1 h, added with 50 μl BSA-PBS-diluted serum (starting with 1/50 dilution), incubated for 4 h, and washed with PBS. Binding was detected following 2 h incubation with BSA-PBS diluted (1/2000) peroxidase-conjugated goat anti-rabbit IgG (γ-chain specific; Accurate Chemical & Scientific Corporation, NY, USA). All incubation steps were performed at 4°C. After washing, color was developed in a substrate solution containing 15 mM o-phenylenediamine and 0.015% H_2O_2 in 0.1 M sodium acetate buffer, pH 5.0, at room temperature. The reaction was stopped after 30 min by addition of 100 μl 0.5 N H_2SO_4, and OD was measured at 450 nm. Non-specific antibody binding (OD value from a well not containing GM1) was subtracted from each measured value. All samples were analyzed in duplicates. Titer values were calculated as the reciprocal of the serum dilution needed to obtain half-maximal antibody binding [14]. Lectin binding was assayed by incubation of 50 μl of

peroxidase-conjugated PNA (20 μg/ml) for 2 h at 4ºC, followed by washing and color reaction development as above.

2.4. Mild periodation treatment.

Microtiter plates adsorbed with KLH were incubated (1 h, 4ºC, dark) with 50 μl of 15 mM sodium periodate in sodium acetate buffer, pH: 4.5. After washing, wells were treated with 50 mM sodium borohydrate in PBS for 30 min. Antibody and lectin binding were assayed as above.

2.5. TLC-immunostaining

A ganglioside mixture containing GA1, GM1, GD1a, GD1b, and GT1b was separated on TLC plates in the running solvent chloroform/ methanol/ aqueous 0.2% $CaCl_2$ (45:45:10) using a tank designed to obtain highly reproducible chromatograms [15]. After air drying, plates were coated by dipping for 90 seconds in a 0.5% solution of poly(isobutyl) methacrylate (Aldrich Chemical Co., Milwaukee, WI, USA) in n-hexane-chloroform (9:1), and air-dried for 10 min. The plates were blocked with BSA-PBS containing 0.05 % Tween 20 (BSA-PBSt) for 1 h, incubated overnight in BSA-PBSt diluted serum (1/1000), washed 3x with PBSt, incubated 2 h with peroxidase-conjugated goat anti-rabbit IgG diluted (1/5000) in BSA-PBSt, and tested for binding. All incubation steps were performed at 4°C. After washing, color was developed in a substrate solution containing 2.8 mM 4-chloro-1-naphthol and 0.01% H_2O_2 in methanol/ 20 mM Tris-HCl buffer, pH 7.4 (1:29) at room temperature. For quantitative studies, spots were measured by densitometry scanning at 590 λ. Usually, one plate was stained with orcinol reagent for chemical detection of gangliosides.

Inhibition of antibody binding to plate bound ganglioside antigen was accomplished by incubating sera with KLH (100 µg/ml) or GA1 (0.1 mM) for 60 min before adding to the plates.

3. Results

After receiving a single dose of immunogen containing BBG and KLH in complete Freund's adjuvant, rabbits begun to produce anti-ganglioside IgG-antibodies, followed by clinical signs of neuropathy one or two weeks later (see supplementary material). Although this association of facts is observed in all the immunized rabbits, their time course was different among them. Figure 1 shows two cases where different time lags between immunization and antibody/disease presence were clearly observed. Anti-ganglioside IgG-antibodies started to be detected four or more weeks after immunization. In contrast, high titers of IgG-antibodies recognizing KLH were already found at week 2 (results not shown). Although these results indicate that different mechanisms could be involved in the induction of both types of antibodies, KLH was necessary to induce anti-ganglioside IgG-antibodies and disease (Table I, groups 1-3). Even more, both components had to be present together in the immunogen because they were ineffective if injected separately (Table I, group 4). Replacing KLH by BSA (a classical protein used as immunological carrier) produced a weaker response of anti-ganglioside IgG-antibodies, and no disease was observed (Table I, group 5). These conditions could be reverted if KLH was injected in a separate site (Table I, group 6), raising antibody titers and inducing clinical signs. These results indicate not only that KLH functions as immunological protein carrier, but also that an immune response against KLH is a requirement for an immune response to gangliosides high enough to induce disease.

Previous reports described that immunization with BBG/KLH induces anti-ganglioside antibodies of the IgM and IgG isotypes [7, 8]. The induced

antibodies recognize gangliosides GA1,GM1and GD1b [16] (Figure 2B). These structurally related gangliosides share the terminal Gal-ß(1,3)-GalNAc disaccharide (Figure 2A). Normal rabbit serum had low levels of anti-ganglioside IgM-antibodies recognizing this terminal [6] (Figure 2Bb, 2Ca, 2Da). This antibody activity was raised by immunization with BBG alone (Figure 2Cb) and, unexpectedly, also in some of the rabbits immunized with KLH (Figure 2Db). No anti-ganglioside IgG-antibody activity was detected, neither in preimmune (normal) sera (Figure 2Bd, 2Cc, 2Dc) nor in BBG immunized rabbits (Figure2Cd). In contrast, KLH-immunized rabbits showed anti-GA1 IgG-antibodies (Figure 2Be). Although this reactivity was very low and sometimes transient, it was clearly detected at 1/20 serum dilutions. Most of normal anti-ganglioside antibody binding reactivity was inhibited by preincubation with GA1, indicating recognition of shared epitopes (Figure 3A). The intensity of the immunospots was variable among different rabbits and, interestingly, a negative correlation between intensity of GA1 spot and time of disease onset was found (Figure 3B).

The protein KLH is highly immunogenic, and very high titers of IgG-antibodies were produced few weeks after immunizations (Figure 4A). KLH is glycosylated [17] and its antigenic reactivity was highly reduced by mild periodation (Figure 4A), a treatment that destroys some terminal sugars like galactose. Peanut agglutinin (PNA) is a lectin that recognizes glycans carrying the terminal Gal-ß(1,3)-GalNAc disaccharide, including GA1 and GM1 gangliosides [18, 19]. PNA also bound KLH (Figure 4B), indicating that KLH contained the terminal disaccharide. As expected, PNA binding was completely eliminated by mild periodation treatment of KLH (Figure 4B).

These results indicated that the immune responses to KLH and to gangliosides could be related and, consequently, a potential cross-reactivity between both antibody activities was studied. For this, sera from sick rabbits were preincubated with soluble KLH before assaying for anti-ganglioside antibodies. As it is shown in Figure 5A, KLH partially inhibited antibody binding to gangliosides. Interestingly, a higher inhibition was obtained at the beginning of the immune response (Figure 5B).

4. Discussion

Gangliosides are ubiquitous molecules present in most of the vertebrate cells and are considered self antigens. The immune response to gangliosides is restricted by self-tolerance,and high affinity monoclonal antibodies can only be elicited in ganglioside-lacking mice [20]. Rabbit serum containing anti-ganglioside antibodies has been produced as a research tool in several laboratories where ganglioside function is studied [21-23], and it is widely known that gangliosides should be mixed with a carrier protein to get antibodies of the IgG isotype. BSA is frequently used as immunological carrier, leading to weak anti-ganglioside IgG antibody responses in rabbits that remain healthy [6] (Table I). The induction of clinical signs of neuropathy by immunization with gangliosides required the presence of KLH in the inoculum. Although this mollusk protein is classically used as immunological carrier protein [24], it cannot be replaced by BSA in order to induce the disease. In addition to its “carrier” function, it appears that KLH is required as immunogen in order to trigger the clinical signs (Table I, group 6).

All the rabbits immunized with BBG and KLH develop the disease, but with differences in the time lags between immunization and emergence of clinical signs. Independently of the lags, anti-ganglioside IgG-antibodies were detected one to two weeks before the signs. These facts indicate that the differences in the lags between immunization and disease onset could be a consequence of differences in the lags of antibody induction. KLH is highly immunogenic, and high titers of antibodies are already induced two weeks after immunization. In contrast, most of the rabbits develop an immune response to gangliosides later. Although these results indicate the involvement of different

mechanisms in the immune response to KLH and gangliosides, KLH immunogenicity is necessary to induce an anti-ganglioside IgG-antibody response high enough to trigger disease.

KLH is a glycoprotein that contains 4 % of carbohydrates [24, 25] and this glycan moiety is the main component of its antigenicity [26] (Figure 4A). One of the glycan structures present in KLH is the so-called “PNA determinant” (terminal Gal-ß(1,3)-GalNAc). This structure is included in the core structure of gangliosides, and is found as a terminal disaccharide in GA1, GM1 and GD1b gangliosides (Figure 2A). We previously described that antibodies induced by immunization with BBG together with a carrier protein recognize these three gangliosides [6, 16]. Fine specificity studies indicated these antibodies are composed by different populations recognizing epitopes formed by terminal Gal-ß(1,3)-GalNAc, with a major or minor contribution of NeuNAc [6, 16]. On the other hand, most of anti-ganglioside IgM-antibodies from normal rabbit recognize epitopes involving terminal Gal-ß(1,3)-GalNAc without participation of NeuNAc. These facts indicated both types of antibodies are related, and that immune antibodies could originate from normal antibodies. In this context, the repertoire of antibodies induced by immunization with KLH could include antibodies recognizing epitopes related to terminal Gal-ß(1,3)-GalNAc. Populations of lymphocytes producing these particular anti-KLH antibodies can be positively selected by immunogenically active gangliosides, thus raising anti-ganglioside antibody levels. At the beginning of the immune response, these anti-ganglioside antibodies will cross-react with KLH. It is expected that later, when more specific anti-ganglioside antibodies are selected, this cross-reactivity would be lost (Figure 5). It appears that the epitopes recognized by

these particular anti-KLH antibodies originating disease-associated anti-ganglioside antibodies are related to the GA1 structure. This is supported by two results: i. Anti-GA1-specific IgG-antibodies (not cross-reacting with GM1/GD1b) were produced in KLH immunized rabbits. ii. Disease triggering was faster in those rabbits containing higher reactivity of normal anti-GA1 IgM-antibodies. However, lymphocytes with this specificity could not be stimulated by GM1/GD1b. Consequently, they should change their specificity in order to be recognized by these gangliosides. We called “binding site drift” to this process of fine specificity modification in the lymphocytes, and it was proposed to explain the origin of anti-ganglioside antibodies in neuropathies [20, 27, 28]. After “drifting”, these new lymphocytes can undergo binding site refinement and affinity maturation, similar to their conventional counterpart. The process would occur at random (drift), a fact that can explain the differences in the immunization/disease lags observed in the sick rabbits (Figure 1).

Conclusion: We can summarize our interpretation of results as follows: during immunization with KLH, lymphocytes that produce antibodies recognizing a GA1-like structure are stimulated. Some of these lymphocytes mutate their surface immunoglobulin binding site, modifying their fine specificity and starting to recognize GM1/GD1b (“drift”). These “drifted” lymphocytes can now be stimulated by immunogenically active GM1/GD1b gangliosides present in the immunogen, triggering the immune response. On the contrary, in the absence of KLH and its associated immune response only lymphocytes producing normally occurring low affinity anti-GM1/GD1b IgM-antibodies can be stimulated, inducing a weak immune response.

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Table I: Immunogen requirements of disease induction.

	Immunogen		Anti-GM1 IgG-antibodies	Clinical signs
Group	Site 1	Site 2		
1	BBG/KLH	-	+	+
2	BBG	-	-	-
3	KLH	-	-	-
4	KLH	BBG	-	-
5	BBG/BSA	-	+/-	-
6	BBG/BSA	KLH	+	+

Rabbits were immunized with an emulsion containing CFA and the stated compounds. Clinical signs included tremor, limb weakness and paralysis. Anti-GM1 antibodies were measured by ELISA: (+) antibody titers between 5700 and 28000 AU; (+/-) antibody titers lower than 1000 AU; (-) non detectable.

Legends to the figures

Figure 1: Time course of disease induction. Rabbits immunized with an emulsion containing BBG, KLH and CFA were weekly weighed and revised to detect appearance of clinical signs. Sera (1/1000 dilutions) were tested for anti-ganglioside IgG-antibodies by TLC-immunostaining (inserts). Arrows indicate the apparition of clinical signs. Results obtained with two rabbits (A and B) with different time lags between immunization and disease triggering is shown.

Figure 2: Anti-ganglioside antibodies characterization. A: Ganglioside structure recognized by rabbit serum antibodies. Representative rabbit sera from groups 1 (B), group 2 (C) and group 3 (D) were analyzed for anti-ganglioside IgM or IgG-antibodies by TLC-immunostaining. Sera were used at 1/20 dilutions, except for IgG determination from group 1 rabbit where serum was used at 1/1000 dilutions. A TLC plate was stained with orcinol reagent for chemical detection of gangliosides.

Figure 3: Normally occurring anti-ganglioside IgM-antibodies in rabbits. Rabbit preimmune sera (1/20 dilution) were assayed for anti-ganglioside IgM-antibodies by TLC immunostaining. Sera were preincubated with and without soluble GA1 (0.1 mM). Results with two representative sera are shown (A). Seven preimmune sera from sick rabbits (Table I, group 1) were analyzed by TLC immunostaining, and spot intensity was quantified by colorimetric scanning. Time of disease onset vs OD values for GA1 spot was plotted (B).

Figure 4: KLH glycan characterization. KLH was adsorbed on ELISA plates and subjected to mild periodation. Treated (●) and non-treated (○) plates were assayed for **A**: Anti-KLH IgG-antibodies present in a group 3 rabbit serum. **B**: PNA lectin binding.

Figure 5: Cross-reactivity between anti-ganglioside and anti-KLH antibodies. A: One thousand dilutions of sera from group 1 rabbits werepreincubated with or without soluble KLH (100 μg/ml). After 1 h at RT, sera were used for TLC-immunostaining (A). Antibody binding was quantified by densitometric scanning of immunospots. Percentages of inhibition obtained in serial serum samples from 3 rabbits are shown (B).

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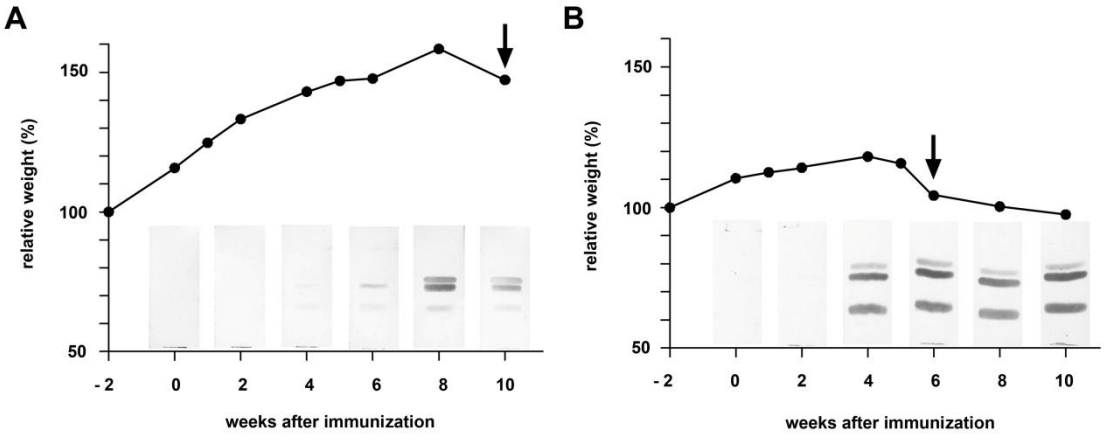


Figure 1

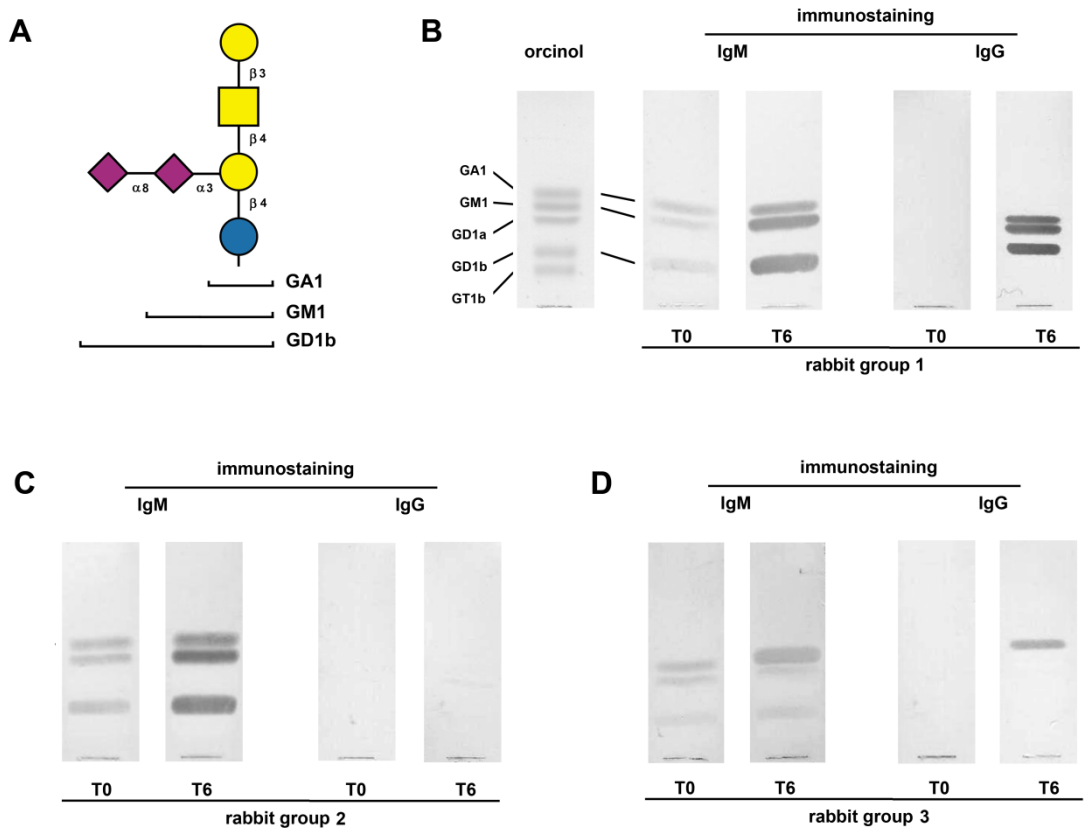


Figure 2

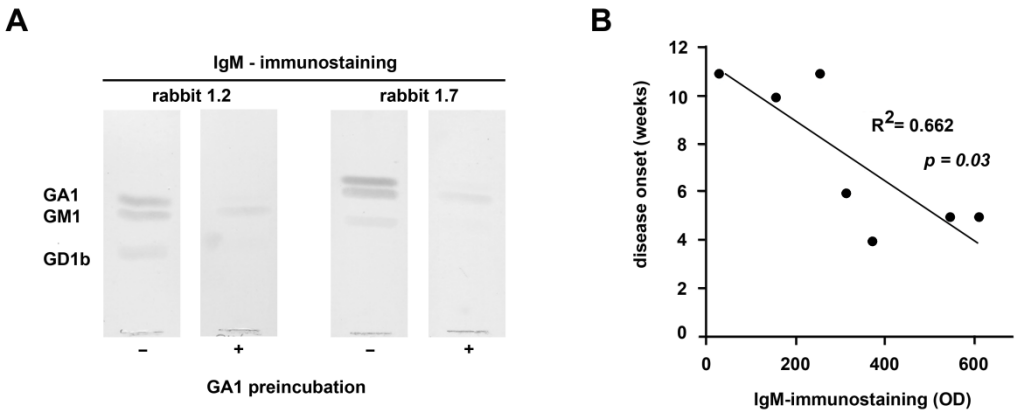


Figure 3

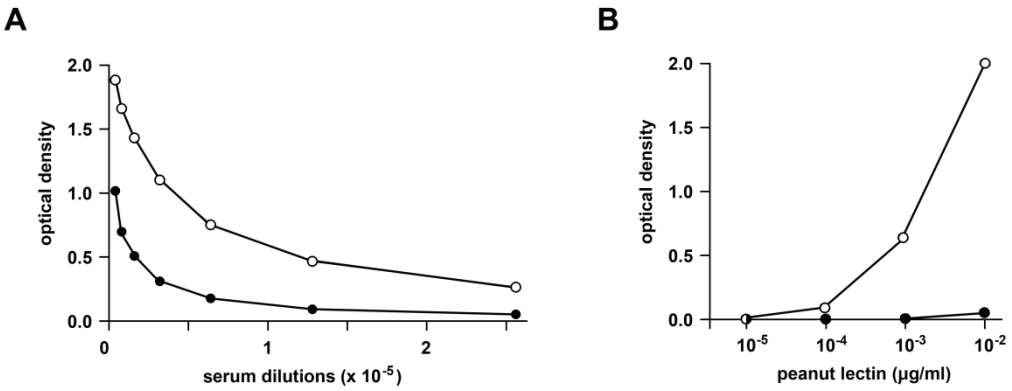


Figure 4

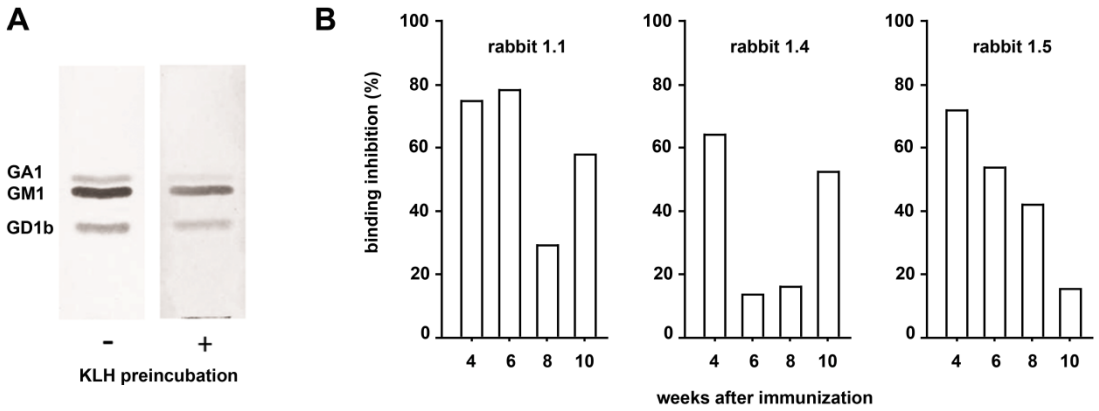
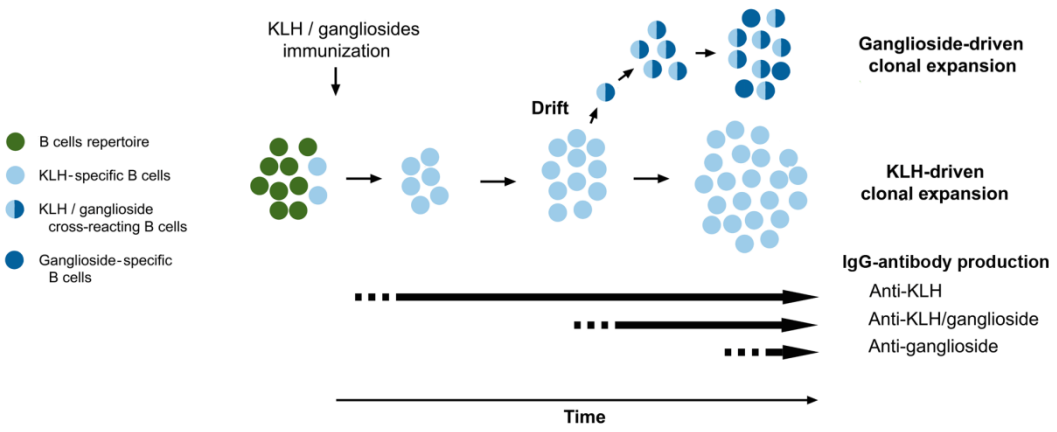


Figure 5



Graphical abstract

Highlights

- Rabbits immunized with gangliosides and KLH develop an experimental neuropathy.
- Anti-ganglioside IgG-antibodies are induced shortly before disease triggering.
- The protein KLH is required both as immunological carrier and as specific immunogen.
- Part of induced anti-ganglioside IgG-reactivity cross-reacts with KLH.
- Disease triggering time lags correlate with preimmune anti-GA1 IgM-antibody levels.
- The terminal glycan Gal-ß(1,3)-GalNAc is proposed as “key” antigenic determinant.