Journal of Neuroimmunology 182 (2007) 116–123

Journal of Neuroimmunology

www.elsevier.com/locate/jneuroim

Leukocyte and complement activation by GM1-specific antibodies is associated with acute motor axonal neuropathy in rabbits

Nina M. van Sorge [a,b,1], Nobuhiro Yuki [c], Marc D. Jansen [a,b], Yukihiro Nishimoto [c], Keiichiro Susuki [c], John H.J. Wokke [b], Jan G.J. van de Winkel [a,d], Leonard H. van den Berg [b,2], W-Ludo van der Pol [b,*,2]

[a] *Department of Immunology, University Medical Center Utrecht, Lundlaan 6, 3584 EA Utrecht, The Netherlands*
[b] *Department of Neurology (G03.228), Rudolf Magnus Institute for Neuroscience, University Medical Center Utrecht, Heidelberglaan 100, 3584CX Utrecht, The Netherlands*
[c] *Department of Neurology and Research Institute for Neuroimmunological Diseases, Dokkyo Medical University School of Medicine, Kitakobayashi 880, Mibu, Shimotsuga, Tochigi 321-0293, Japan*
[d] *Genmab, Yalelaan 60, 3584 CM, Utrecht, The Netherlands*

Received 14 July 2006; received in revised form 9 October 2006; accepted 9 October 2006

Abstract

Acute motor axonal neuropathy (AMAN) in humans is associated with the presence of GM1-specific antibodies. Immunization of rabbits with GM1-containing ganglioside mixtures, purified GM1, or *Campylobacter jejuni* lipo-oligosaccharide exhibiting a GM1-like structure elicits GM1-specific antibodies, but axonal polyneuropathy only occurs in a subset of animals. This study aimed to dissect the molecular basis for the variable induction of AMAN in rabbits. Therefore, we analyzed the pro-inflammatory characteristics of GM1-specific antibodies in plasma samples from ganglioside-immunized rabbits with and without neurological deficits. GM1-specific plasma samples from all rabbits with AMAN were capable of activating both complement and leukocytes, in contrast to none of the plasma samples from rabbits without paralysis. Furthermore, GM1-specific IgG-mediated activation of leukocytes was detected before the onset of clinical signs. These data suggest that AMAN only occurs in rabbits that develop GM1-specific antibodies with pro-inflammatory properties.

Keywords: Acute motor axonal neuropathy; Complement; FcγR; Autoantibody; Ganglioside

1. Introduction

Guillain–Barré syndrome (GBS) is an immune-mediated polyradiculoneuropathy, presumably triggered by autoantibodies, complement, and leukocytes (Kieseier et al., 2004). Acute motor axonal neuropathy (AMAN) is a GBS subtype, characterized by axonal degeneration, primary motor deficits, and the presence of GM1-specific antibodies of all major isotypes (IgM, IgG, IgA) (Koga et al., 1999; Ogawara et al., 2000). Several lines of evidence support a role of GM1-specific antibodies in the pathogenesis of AMAN. Firstly, immunohistochemistry showed that GM1 is highly expressed in motor nerves and axons (Gong et al., 2002). Secondly, intraneural injection of rabbit anti-GM1 plasma or purified IgG containing GM1-specific antibodies from patients with AMAN in rat sciatic nerves induces predominantly axonal degeneration (Yuki et al., 2002).

Immunization of rabbits with ganglioside mixtures or isolated GM1 generally elicits GM1-specific IgG antibodies (Lopez et al., 2002; Yuki et al., 2001). However, signs of axonal neuropathy with strong clinical, pathological, and

* Corresponding author. Tel.: +31 30 2507975; fax: +31 30 2542100.
E-mail address: W.L.vanderPol@umcutrecht.nl (W.-L. van der Pol).
[1] Current address: Department of Infectious Diseases and Immunology, Utrecht University, Yalelaan 1, PO box 80.165, 3508 TD, Utrecht, The Netherlands.
[2] These authors contributed equally to this work.

0165-5728/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneuroim.2006.10.005

neurophysiological resemblance to human AMAN are only induced when specific immunization conditions are used (Susuki et al., 2003, 2004; Yuki et al., 2001). Under such conditions approximately 80% of immunized rabbits develop axonal neuropathy (Nishimoto et al., 2004). This suggests that the presence of GM1-specific antibodies is, in itself, not enough to initiate disease.

We hypothesized that pathogenicity of GM1-specific antibodies is determined by their pro-inflammatory properties, i.e. their capacity to activate complement, inflammatory cells through antibody receptors (FcR), or both. The capacity of (auto)antibodies to exert such pro-inflammatory functions is determined by antibody subclass, avidity, specificity, and epitope density in the target tissue (Goodfellow et al., 2005; Romero-Steiner et al., 1999). Although previous studies demonstrated that human ganglioside-specific Ig can activate complement (Halstead et al., 2004; Zhang et al., 2004) and leukocytes (van Sorge et al., 2003), it is unknown whether these antibody characteristics are critical for the development of AMAN. Therefore, antibody-induced leukocyte and complement activation were compared using plasma samples from ganglioside-immunized rabbits with and without paralysis, but with similar GM1-specific IgG titers.

2. Materials and methods

2.1. Rabbits and plasma samples

Forty-two rabbit plasma samples were used in this study. Immunization protocols of Japanese white (JW/CSK; $n=20$) and New Zealand white (NZW) rabbits ($n=2$) with a bovine brain ganglioside mixture (including GM1) have been described in detail (Lopez et al., 2002; Yuki et al., 2001). Plasma samples ($n=41$) were obtained during previously published studies (Lopez et al., 2002; Nishimoto et al., 2004; Yuki et al., 2001). One plasma sample was additionally purchased from Calbiochem (San Diego, CA).

Thirteen plasma samples from ganglioside-immunized JW/CSK rabbits with flaccid paralysis were obtained at onset of weakness (Table 1, *Rabbit 1–13*)(Yuki et al., 2001). Poorest functional grade of the AMAN rabbits was defined as clinical severity score for limb weakness following ganglioside immunization: 0=normal; 1=showing weakness of hind limbs; 2=showing mild weakness of all four limbs, but able to walk; 3=showing moderate weakness of all four limbs, and unable to walk; 4=showing severe weakness of all four limbs, which are spread out; 5=dead (Yuki et al., 2001). Mild disease course was defined as grades 1–2, severe disease as grades 3–4. None of the animals died. In addition, a total of 19 plasma samples, drawn at one-week intervals (ranging from week 2 after immunization until disease onset), were obtained from 5 of these rabbits (Table 1, *Rabbits 6*, *8*, *10*, *11*, and *13*).

Ten plasma samples were obtained from ganglioside-immunized rabbits, which did not develop flaccid paralysis (Table 1, *Rabbits 14–23*). Seven of these plasma samples were obtained from JW/CSK rabbits 190 to 197 days after first immunization (Nishimoto et al., 2004). These rabbits were immunized with ganglioside mixtures using exactly the same protocol which induced paralysis in rabbits 1–13. Two plasma samples were obtained from NZW rabbits 63 and 254 days after ganglioside immunization (Table 1, *Rabbits 21* and *22*) (Lopez et al., 2002). One plasma sample (Table 1, *Rabbit 23*) was purchased from Calbiochem (San Diego, CA).

Table 1
Characteristics of ganglioside-immunized rabbits

Rabbit[1]	Original code[2]	Strain[3]	Anti-GM1 IgG titer	Poorest functional grade
1	Cr-1	JW/CSK	4000	1
2	Cr-2	JW/CSK	4000	1
3	Cr-7	JW/CSK	32,000	2
4	Cr-10*	JW/CSK	32,000	3
5	Cr-3	JW/CSK	32,000	4
6	Cr-4*	JW/CSK	128,000	4
7	Cr-5*	JW/CSK	64,000	4
8	Cr-6*	JW/CSK	64,000	4
9	Cr-8	JW/CSK	16,000	4
10	Cr-9	JW/CSK	64,000	4
11	Cr-11	JW/CSK	16,000	4
12	Cr-12	JW/CSK	8000	4
13	Cr-13	JW/CSK	2000	4
14		JW/CSK	32,000	0
15		JW/CSK	32,000	0
16		JW/CSK	32,000	0
17		JW/CSK	32,000	0
18		JW/CSK	16,000	0
19		JW/CSK	8000	0
20		JW/CSK	2000	0
21		NZW	3200	0
22		NZW	12,800	0
23		unknown	4000	0

[1]Samples were obtained from experiments in previous studies. Rabbits 1–13 (Yuki et al., 2001); rabbits 14–20 (Nishimoto et al., 2004); rabbits 21 and 22 (Lopez et al., 2002). [2] Rabbit codes as previously published (Yuki et al., 2001); [3] JW/CSK=Japanese white rabbit; NZW=New Zealand white rabbit. * High affinity anti-GM1 IgG antibodies present at disease onset (Comín et al, 2006).

2.2. GM1-specific IgG ELISA

Assessment of anti-GM1 IgG titers in rabbit plasma samples was performed as described previously (Yuki et al., 2001). Shortly, 5 pmol of GM1 was placed in individual wells of ELISA plates. Plasma samples were serially diluted starting at 1:500 and incubated overnight at 4 °C. Peroxidase-conjugated anti-rabbit IgG was added for 2 h at room temperature, and developed with *o*-phenylenediamine.

2.3. Complement activation assay

Complement activation by GM1-specific antibodies in rabbit plasma samples was determined as follows. Ninety-six well plates (NUNC, Polysorp®, Roskilde, Denmark) were coated with 0.5 μg of GM1 (Calbiochem, San Diego, CA) and saturated with PBS 1% BSA for 4 h at room temperature.

Rabbit plasma samples were serially diluted in triplicate (starting 1:1000) in PBS 1% BSA and incubated overnight at 4 °C. Wells coated with a concentration range of purified rabbit IgG (Sigma; 100–0.1 μg/ml) and BSA were used as positive and negative controls, respectively. Plates were washed 6 times with PBS and incubated with 2% baby rabbit serum (Serotec, Oxford, UK) in PBS as a complement source (37 °C, 30 min), or PBS alone as a negative control. Plates were washed 6 times with PBS before addition of goat anti-rabbit C3b and C3c specific antiserum (Nordic; 1:10,000 dilution) for 1 h at room temperature. Plates were washed 6 times with PBS, and HRP-labeled rabbit anti-goat IgG (Jackson Immunoresearch, UK; 1:1000 dilution) was added. Finally, after washing 6 times with PBS, plates were developed using ABTS® and optical densities were determined using an ELISA reader (Multiscan RC, Thermo Labsystems, 405 nm). Complement activation was considered positive if 1) the OD at a 1:1000 dilution was higher than the mean plus two times the standard deviation of OD obtained with a serum pool from pre-immunized rabbits ($n=10$, data not shown), and 2) if there was a gradual decrease in C3b/c deposition with increasing plasma dilutions. For quantification of complement activation, the optical density of GM1-coated wells, after subtraction of background values, at a 1:2000 dilution was used, which reflects the linear phase of the signal in all plasma samples.

2.4. Leukocytes

Polymorphonuclear granulocytes (PMN) were freshly isolated for each individual experiment from heparinized venous blood of healthy volunteers using Ficoll-Histopaque (Sigma, St. Louis, MO) gradient centrifugation. Remaining erythrocytes were removed by hypotonic lysis. PMN were washed and resuspended in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal-calf serum, at a concentration of 1×10^7 cells/ml. Cell viability was >99% as determined by Trypan blue exclusion.

2.5. Leukocyte degranulation assay

GM1-specific Ig-induced leukocyte degranulation was assessed as described before (van Sorge et al., 2003). Shortly, 96 well plates (NUNC, Polysorp®) were coated with 0.5 μg GM1, blocked with PBS 1% BSA, and incubated overnight at 4 °C with different dilutions (starting 1:20) of rabbit plasma samples in triplicate as described above. After washing 6 times with PBS, 1.5×10^5 freshly isolated PMN from healthy volunteers, suspended in 150 μl Hank's balanced saline solution (Gibco) 1% BSA, were added to the wells, and incubated for 1 h at 37 °C. After centrifugation, supernatants were collected and stored at −80 °C until further testing. Supernatants from wells coated with GM1 containing PMN, but without rabbit plasma,

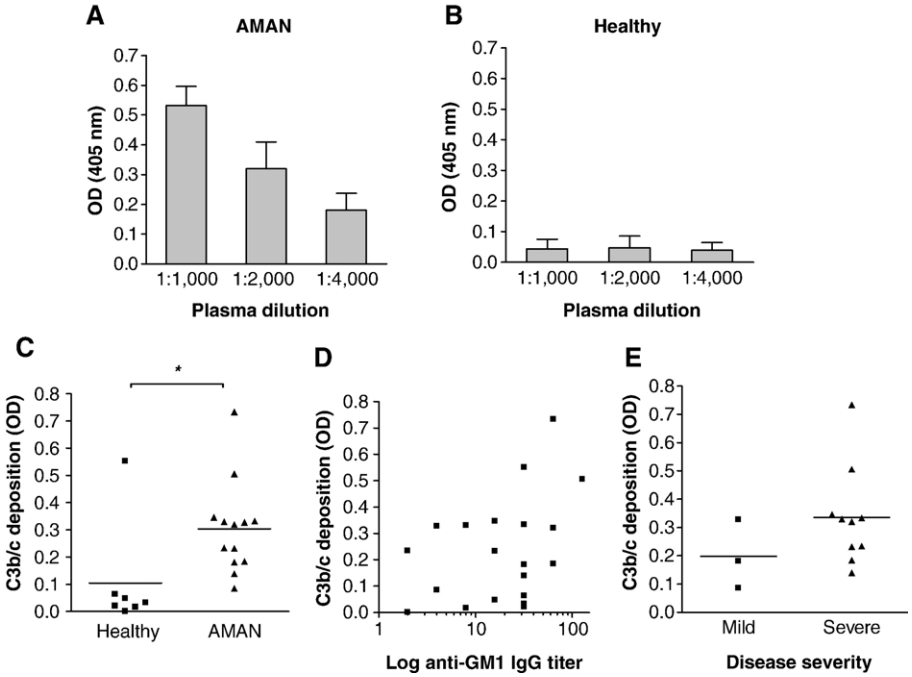

Fig. 1. AMAN is associated with complement activating capacity of GM1-specific antibodies. GM1-specific Ig in plasma from rabbits with (A) AMAN, but not from (B) ganglioside-immunized healthy rabbits, activates complement. Representative results from one rabbit with AMAN and one healthy rabbit are shown. Each rabbit plasma sample was tested twice in triplicate; bars represent mean±SD from one experiment. (C) GM1-specific Ig in plasma samples from 20 ganglioside-immunized rabbits, seven from healthy rabbits and 13 from AMAN rabbits, differ in their efficacy to activate complement (* $p<0.01$, Mann-Whitney U-test). Each dot represents one rabbit plasma sample at a 1:2000 dilution. Bars indicate mean of complement activation. Representative results from two independent experiments are shown. (D) GM1-specific complement activation does not correlate with anti-GM1 IgG titer in rabbits ($R^2=0.35$, $p=0.14$). Each dot represents complement activation by a single rabbit plasma sample at a 1:2000 dilution. (E) Complement activation by plasma samples from rabbits with severe and mild AMAN does not differ significantly. Each dot represents one plasma sample from the AMAN rabbits at a 1:2000 dilution. Bars represent means.

were used as negative controls, whereas PMN incubated with formyl-met-leu-phe (Sigma) in the presence of cytochalasin B (Sigma), and PMN-lysates served as positive controls. Pilot experiments showed that monomeric rabbit IgG directly coated on surfaces of 96 well plates induced degranulation of human leukocytes (data not shown), confirming that human leukocytes can be used to test functionality of rabbit IgG *in vitro*.

Monoclonal antibodies IV.3 F(ab') fragments and 3G8 F $(ab')_2$ fragments (both Medarex, Annandale, NJ) directed against PMN IgG receptors (FcγR) IIa (CD32a) and FcγRIIIb (CD16b), respectively, were used for blocking experiments. PMN were incubated with 50 μg/ml of IV.3 and 3G8 for 30 min at 4 °C prior to degranulation experiments.

2.6. Lactoferrin sandwich ELISA

Lactoferrin is a marker for FcγR-mediated specific granule release by leukocytes. Lactoferrin concentrations in harvested supernatants were determined using a lactoferrin sandwich ELISA as described before (van Spriel et al., 2001). Six fixed lactoferrin (Sigma) concentrations (in triplicate) were used to construct standard curves in each ELISA plate. Degranulation responses above background were defined as lactoferrin concentrations that exceeded at least 1.5 times the background level at the highest plasma concentration, in addition to a concentration dependent decrease upon plasma dilution (van Sorge et al., 2003). For select experiments, degranulation responses were quantified by estimating the 'best fitting' curves of serial serum dilutions using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). The ratio of the area under the curve (AUC) of wells with (AUC_{GM1}) and without ganglioside (AUC_{BSA}; background level) was used to quantify degranulation efficacy. As expected, lactoferrin release quantitatively showed donor-to-donor variation. Therefore, PMN from a single donor were used to compare degranulation responses induced by different rabbit plasma samples.

2.7. Analysis

SPSS version 12.0.1 for Windows was used for statistical analysis. Antibody titers of ganglioside-immunized healthy and AMAN rabbits were compared by Student's *t* tests. Fisher's exact test was used for comparison of the numbers of complement- and leukocyte-activating plasma samples from healthy and AMAN rabbits. Mann-Whitney *U*-test and

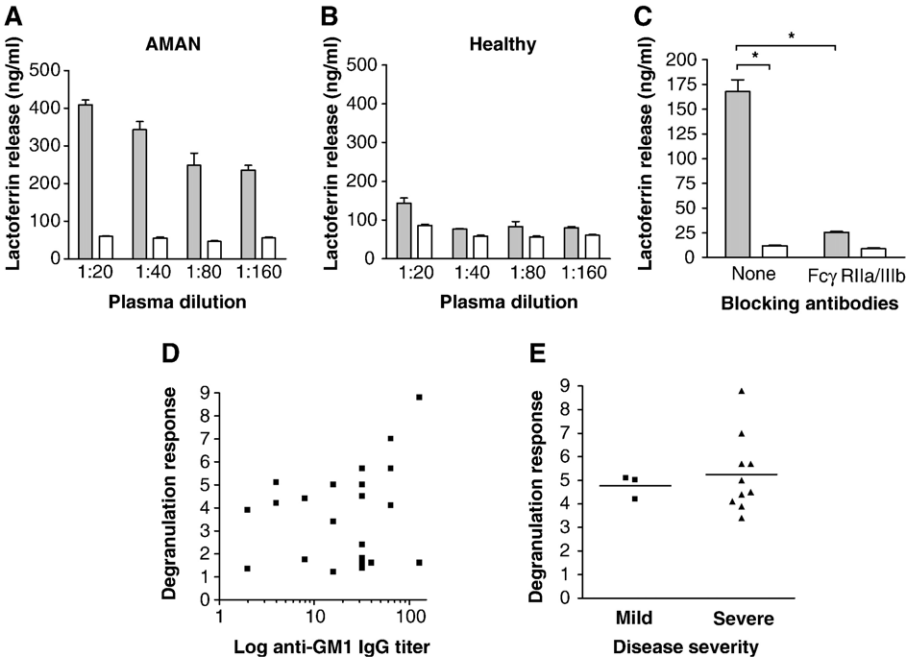

Fig. 2. AMAN is associated with leukocyte-activating capacity of GM1-specific IgG through FcγR crosslinking. GM1-specific IgG in plasma from (A) ganglioside-immunized rabbits with AMAN, but not from (B) ganglioside-immunized healthy rabbits, induces leukocyte degranulation, as measured by lactoferrin release in cell supernatants. Representative results from one rabbit with AMAN and one healthy rabbit are shown. Grey bars represent results from GM1-coated wells, and white bars the background signal. Each rabbit plasma sample was tested twice in triplicate using different PMN donors; bars represent mean±SD from one experiment. (C) Leukocyte degranulation induced by plasma from rabbits with AMAN is FcγR-dependent. Pooled plasma from three rabbits with AMAN (*Rabbits 1, 3*, and *5*) was used to test the effect of FcγRII and FcγRIII-specific blocking monoclonal antibodies on GM1-specific IgG-induced leukocyte degranulation (plasma dilution 1:20). Grey bars represent results from GM1-coated wells, and white bars the background signal. The experiment was performed three times in triplicate using different PMN donors; bars represent mean±SD from one representative experiment. * $p<0.05$. (D) Magnitude of GM1-specific leukocyte degranulation induced by plasma from ganglioside-immunized rabbits does not correlate with anti-GM1 IgG titers ($R^2=0.22$, $p=0.33$). Each dot represents the degranulation response of one rabbit plasma sample. (E) Magnitude of degranulation responses does not differ between mild (grade 1 and 2) and severe (grade 3 and 4) disease course in AMAN rabbits (independent samples *t*-test, $p>0.5$). Each dot represents the degranulation response of one rabbit plasma sample. Bars represent means.

2 samples *t*-test were used to test quantitative differences in complement activation and degranulation responses, respectively, by GM1-specific antibody-containing plasma samples from healthy and AMAN rabbits. Spearman's correlation analysis was used to test correlation between antibody titers in rabbit plasma samples and leukocyte degranulation, or complement activation. Statistical significance was accepted at $p<0.05$.

3. Results

3.1. GM1-specific IgG titers

GM1-specific IgG titers were high in all plasma samples (Table 1), and did not differ significantly between rabbits with and without paralysis.

3.2. Complement activation by GM1-specific antibodies is associated with AMAN

Plasma samples from ganglioside-immunized JW/CSK rabbits with and without paralysis were used in complement activation experiments. Plasma from ganglioside-immunized healthy rabbits and rabbits with AMAN differed significantly in their capacity to activate complement, as shown in Fig. 1A, B. Two out-of-seven (29%) plasma samples from healthy rabbits and 13 out-of-13 (100%) plasma samples from the AMAN rabbits activated complement ($p=0.001$, Fisher's exact test). Moreover, C3b/c deposition was significantly higher using plasma samples from the AMAN rabbits as compared to healthy rabbits (Fig. 1C, $p<0.01$). Complement activation by GM1-specific antibodies did not correlate significantly with GM1-specific IgG titers (Fig. 1D), nor with disease severity in the AMAN rabbits (Fig. 1E).

3.3. GM1-specific IgG in plasma samples from AMAN rabbits activates leukocytes through FcγR crosslinking

Next, we evaluated leukocyte activation by GM1-specific antibodies in rabbit plasma by measuring their capacity to induce leukocyte degranulation responses. For these experiments, a total of 23 rabbit plasma samples were available (Table 1). Leukocyte degranulation higher than background was observed with all plasma samples from the AMAN rabbits (13/13, 100%), but with only 2 out-of-10 plasma samples from ganglioside-immunized healthy rabbits (20%; $p<0.001$, Fisher's exact test). Representative results of leukocyte degranulation responses induced by GM1-specific plasma samples from the AMAN rabbits and healthy rabbits are shown in Fig. 2A, B. Experiments using pooled plasma from 3 AMAN rabbits (*Rabbits 1*, *3* and *5*; Table 1) showed that the presence of both FcγRII- and FcγRIII-blocking antibodies virtually abrogated leukocyte degranulation responses (Fig. 2C). This confirms that leukocyte degranulation is mediated by FcγR crosslinking. GM1-specific IgG titers did not correlate significantly with degranulation activity (Fig. 2D), nor did degranulation responses differ between rabbits with mild and severe disease (Fig. 2E).

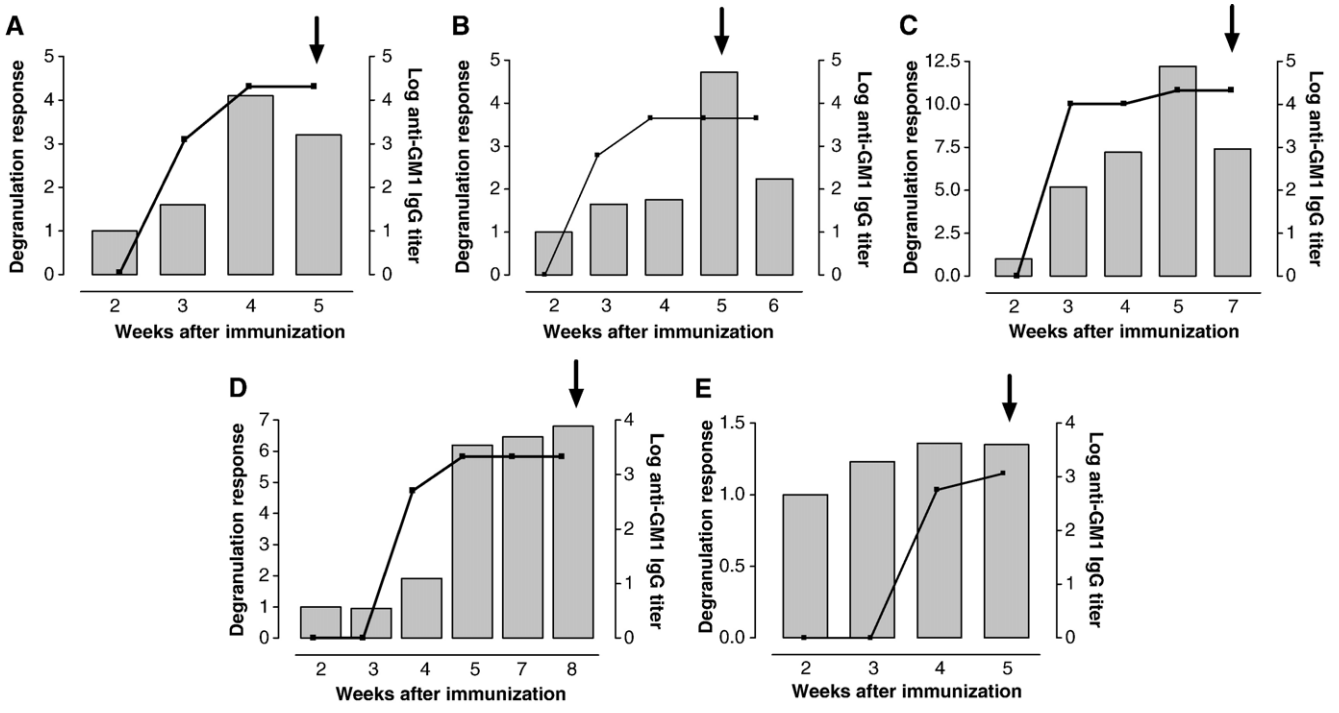

Fig. 3. GM1-specific IgG-induced leukocyte degranulation is associated with onset of AMAN in rabbits. Leukocyte activation by GM1-specific IgG-containing plasma from ganglioside-immunized rabbits that developed AMAN is highest 1 week prior to, or at onset of weakness. Bars represent relative increase of degranulation responses compared to the first plasma sample drawn at 2 weeks (left *y*-axis), lines represent log anti-GM1 IgG titers (right *y*-axis). Arrows mark the onset of clinical symptoms. A, *Rabbit 6* (*Cr-4*); B, *Rabbit 8* (*Cr-6*); C, *Rabbit 10*; D, *Rabbit 11*; E, *Rabbit 13*.

Overall, all plasma samples from the AMAN rabbits, but none of the plasma samples from ganglioside-immunized healthy rabbits induced both leukocyte and complement activation ($p<0.001$, Fisher's exact test).

3.4. Leukocyte activating capacity of GM1-specific antibodies is associated with onset of flaccid paralysis

To investigate the pro-inflammatory function of GM1-specific IgG in relation to the onset of limb weakness, serially drawn plasma samples from 5 AMAN rabbits were used (*Rabbits 6*, *8*, *10*, *11*, and *13*; Table 1). GM1-specific IgM titers were detectable 2–3 weeks after initial immunization, and IgG titers approximately 1 week later (Yuki et al., 2001). Pro-inflammatory activity of GM1-specific IgG, as measured by degranulation-inducing capacity of plasma, could be detected after the appearance of significant IgG titers, reaching peak levels 1 week before, or coinciding with, the onset of clinical symptoms (Fig. 3A–E).

4. Discussion

Immunization of rabbits with gangliosides generally elicits GM1-specific IgM and IgG antibodies (Lopez et al., 2002; Yuki et al., 2001), but flaccid paralysis is only induced under specific immunization conditions (Susuki et al., 2004; Yuki et al., 2001). We hypothesized that the pro-inflammatory properties of GM1-specific antibodies, i.e. their capacity to activate complement or inflammatory cells, are prerequisite for the occurrence of paralysis. Indeed, leukocyte and complement activation were both triggered by GM1-specific antibodies in plasma samples from the AMAN rabbits, but by none of the plasma samples from healthy rabbits, despite similar GM1-specific IgG titers. This strongly suggests that pro-inflammatory activity of GM1-specific antibodies reflects antibody pathogenicity. Furthermore, leukocyte activation by plasma samples from AMAN rabbits was detected before the onset of paralysis, suggesting that ganglioside-specific antibodies with pro-inflammatory activity are not the result of an immune response induced by general nerve damage occurring in the course of AMAN.

PMN were used as effector cells, since they abundantly express FcγR and can be readily harvested in large numbers. Furthermore, activation of granulocytes and monocytes by ganglioside-specific antibodies showed a high degree of correlation in previous experiments (van Sorge et al., 2003). In addition, FcγRIIa, the most important FcγR subclass on PMN for triggering degranulation, is constitutively expressed on PMN, monocytes and macrophages. Although the role of PMN in the pathogenesis of GBS is unknown, it is generally assumed that mononuclear phagocytes predominate the late phases of leukocyte-mediated inflammation. Correspondingly, histological studies of nerves from AMAN patients and rabbits show such mononuclear infiltrates (McKhann et al., 1993; Susuki et al., 2003). However, PMN may initiate or contribute to the early phases of other inflammatory reactions, and may have disappeared from nerves at the time of autopsy.

The apparent difference in biological activity of GM1-specific antibodies in plasma from the AMAN rabbits compared to plasma from rabbits without signs of neuropathy may be explained by crucial differences in antibody avidity or specificity. High avidity of antibodies is generally associated with the capacity to induce pro-inflammatory effector functions, including activation of complement and leukocytes (Romero-Steiner et al., 1999). Indeed, avidity of experimentally elicited GM1-specific IgG from rabbits without signs of neuropathy was significantly lower than similar antibodies in sera from patients with GBS (Lopez et al., 2002). Furthermore, a recent study showed that affinity of GM1-specific IgG increased at the onset of disease in rabbits with experimentally induced AMAN (Comín et al., 2006). Two rabbit plasma samples were also included in this study (Rabbits Cr-4 and Cr-6). Leukocyte-activation by plasma samples from Rabbit Cr-6 with AMAN (Fig. 3B) coincided with a previously reported increase in GM1-specific antibody affinity at onset of disease (Comín et al., 2006). Plasma samples from Rabbit Cr-4 with AMAN (Fig. 3A) induced degranulation 1 week before disease onset, i.e. before an increase in affinity could be measured. However, degranulation capacity was accompanied by a log increase of the GM1-specific IgG titer, suggesting that increasing titer and increasing affinity may both contribute to antibody functionality. Furthermore, two rabbit plasma samples with high anti-GM1 antibody titers (*Rabbits 21* and *22*; Table 1), but documented low avidity (Lopez et al., 2002), did not activate leukocytes. Alternatively, antibody fine specificity may determine antibody pathogenicity. Development of sensory ataxic neuropathy in GD1b-immunized rabbits was associated with antibody monospecificity (Kusunoki et al., 1999). The two features may also be inter-related, since it was reported that cross-reactive GM1 antibodies displayed lower affinity than monospecific anti-GM1 antibodies (Deisenhammer et al., 1996).

Onset and clinical course of experimentally induced AMAN may be variable, and may be explained by genetic heterogeneity of rabbits. Onset of AMAN in the rabbits used for this study ranged from 35 to 57 days (Yuki et al., 2001). Plasma samples from animals that did not develop AMAN despite six months of repeated inoculations, were used as a control group. Because of the limited variability in time until onset, we feel that it is unlikely that these animals would have developed AMAN at a later stage. The data from the longitudinal study suggest that changes in functionality of ganglioside-specific antibodies would precede or coincide with onset of disease.

The relative importance of FcγR-mediated leukocyte activation and complement for induction of nerve damage is currently unknown, although animal models suggest that inflammation is induced by coexertion of complement and leukocytes (Ravetch, 2002). Similarly, complement deposits and infiltrates of phagocytes are both detected in nerve autopsies from patients and rabbits with AMAN (Hafer-

Macko et al., 1996; Susuki et al., 2003, 2006). The nerve-damaging effect of complement activation, and – more specifically – formation of the membrane-attack complex, was convincingly demonstrated *in vitro* (Bullens et al., 2000; Halstead et al., 2004) and *in vivo* by passive transfer of GQ1b-specific monoclonal IgM in mice (Halstead et al., 2004). Importantly, these mice did not develop overt signs of neuropathy. The contribution of FcγR to the development of AMAN has, as yet, not been evaluated *in vivo*. However, in mice with Sandhoff disease, which is characterized by lysosomal accumulation of GM2 and the presence of GM2-specific antibodies, disease course was significantly attenuated by disruption of FcγR (Yamaguchi et al., 2004). In addition, association studies show that FcγR polymorphisms, which determine efficacy of IgG-induced leukocyte activation, are among the rare genetic markers for GBS severity (van Sorge et al., 2005). Although 4 out-of-10 plasma samples from ganglioside-immunized healthy rabbits triggered either complement or leukocyte activation, none of these plasma samples induced both leukocyte and complement activation. This may imply that simultaneous activation of both leukocytes and complement is required for the induction of AMAN, or that GM1-specific IgG from rabbits with AMAN are generally more efficient in inducing pro-inflammatory functions.

Activation of the innate immune system may constitute an additional prerequisite for the occurrence of AMAN, since passive transfer of ganglioside-specific monoclonal IgG does not always induce AMAN in experimental animals (Sheikh et al., 2004). Pro-inflammatory cytokines may play a role in this respect, since they may initiate breakdown of the blood-nerve barrier, and facilitate access of pathogenic antibodies to nerves (Créange et al., 1997).

It was reported previously that the peak of ganglioside-specific autoantibody titers in patients with AMAN does not always coincide with onset of disease (Press et al., 2001). This may be explained by the fact that titer does not accurately reflect pro-inflammatory functions of these antibodies. Similarly, titers of GM1-specific IgG in rabbits did not correlate with complement or leukocyte activating capacity. Only when rabbits with GM1-specific IgG but without paralysis were excluded from this analysis, GM1-IgG titer and leukocyte activation showed a significant correlation ($R^2=0.59$, $p=0.03$). It is therefore tempting to speculate that not all GM1-specific antibodies in plasma from patients with AMAN are equally pathogenic, but that inflammation is induced by a subset of autoantibodies characterized by pro-inflammatory capacity.

A correlation between the magnitude of complement or leukocyte activation and disease severity in paralyzed rabbits was not observed. Because the number of tested animals was small, we cannot exclude that the current study is underpowered for the detection of such associations. Alternatively, pro-inflammatory activity of rabbit plasma may predict long-term outcome (or the extent of axonal damage), rather than severity. Long-term outcome in rabbits was not assessed systematically, but longitudinal studies on the association of the pro-inflammatory function of patient sera and outcome have recently been initiated.

Acknowledgements

The authors would like to thank Dr. P. Westers (Center for Biostatistics, Utrecht University, The Netherlands) for his help and advice with statistical analysis. The authors wish to thank Dr. P. H. Lopez and Dr. G. A. Nores, Cordoba, Argentina, for kindly providing serum samples of *Rabbits 21* and *22*, and Dr. Jos A.G. van Strijp, Dr. Kok P. van Kessel, and Prof. Dr. J. P. van Putten for their valuable suggestions.

References

Bullens, R.W., O'Hanlon, G.M., Goodyear, C.S., Molenaar, P.C., Conner, J., Willison, H.J., Plomp, J.J., 2000. Anti-GQ1b antibodies and evoked acetylcholine release at mouse motor endplates. Muscle Nerve 23, 1035–1043.

Comín, R., Yuki, N., Lopez, P.H., Nores, G.A., 2006. High affinity of anti-GM1 antibodies is associated with disease onset in experimental neuropathy. J. Neurosci. Res. 84, 1085–1090.

Créange, A., Barlovatz-Meimon, G., Gherardi, R.K., 1997. Cytokines and peripheral nerve disorders. Eur. Cytokine Netw. 8, 145–151.

Deisenhammer, F., Keir, G., Pfausler, B., Thompson, E.J., 1996. Affinity of anti-GM1 antibodies in Guillain–Barré syndrome patients. J. Neuroimmunol. 66, 85–93.

Gong, Y., Tagawa, Y., Lunn, M.P., Laroy, W., Heffer-Lauc, M., Li, C.Y., Griffin, J.W., Schnaar, R.L., Sheikh, K.A., 2002. Localization of major gangliosides in the PNS: implications for immune neuropathies. Brain 125, 2491–2506.

Goodfellow, J.A., Bowes, T., Sheikh, K., Odaka, M., Halstead, S.K., Humphreys, P.D., Wagner, E.R., Yuki, N., Furukawa, K., Plomp, J.J., Willison, H.J., 2005. Overexpression of GD1a ganglioside sensitizes motor nerve terminals to anti-GD1a antibody-mediated injury in a model of acute motor axonal neuropathy. J. Neurosci. 25, 1620–1628.

Hafer-Macko, C., Hsieh, S.T., Li, C.Y., Ho, T.W., Sheikh, K., Cornblath, D.R., McKhann, G.M., Asbury, A.K., Griffin, J.W., 1996. Acute motor axonal neuropathy: an antibody-mediated attack on axolemma. Ann. Neurol. 40, 635–644.

Halstead, S.K., O'Hanlon, G.M., Humphreys, P.D., Morrison, D.B., Morgan, B.P., Todd, A.J., Plomp, J.J., Willison, H.J., 2004. Anti-disialoside antibodies kill perisynaptic Schwann cells and damage motor nerve terminals via membrane attack complex in a murine model of neuropathy. Brain 127, 2109–2123.

Kieseier, B.C., Kiefer, R., Gold, R., Hemmer, B., Willison, H.J., Hartung, H.P., 2004. Advances in understanding and treatment of immune-mediated disorders of the peripheral nervous system. Muscle Nerve 30, 131–156.

Koga, M., Yuki, N., Hirata, K., 1999. Subclass distribution and the secretory component of serum IgA anti-ganglioside antibodies in Guillain–Barré syndrome after *Campylobacter jejuni* enteritis. J. Neuroimmunol. 96, 245–250.

Kusunoki, S., Hitoshi, S., Kaida, K., Arita, M., Kanazawa, I., 1999. Mono-specific anti-GD1b IgG is required to induce rabbit ataxic neuropathy. Ann. Neurol. 45, 400–403.

Lopez, P.H., Villa, A.M., Sica, R.E., Nores, G.A., 2002. High affinity as a disease determinant factor in anti-GM1 antibodies: comparative characterization of experimentally induced vs. disease-associated antibodies. J. Neuroimmunol. 128, 69–76.

McKhann, G.M., Cornblath, D.R., Griffin, J.W., Ho, T.W., Li, C.Y., Jiang, Z., Wu, H.S., Zhaori, G., Liu, Y., Jou, L.P., et al., 1993. Acute motor axonal neuropathy: a frequent cause of acute flaccid paralysis in China. Ann. Neurol. 33, 333–342.

Nishimoto, Y., Koga, M., Kamijo, M., Hirata, K., Yuki, N., 2004. Immunoglobulin improves a model of acute motor axonal neuropathy by preventing axonal degeneration. Neurology 62, 1939–1944.

Ogawara, K., Kuwabara, S., Mori, M., Hattori, T., Koga, M., Yuki, N., 2000. Axonal Guillain–Barré syndrome: relation to anti-ganglioside antibodies and *Campylobacter jejuni* infection in Japan. Ann. Neurol. 48, 624–631.

Press, R., Mata, S., Lolli, F., Zhu, J., Andersson, T., Link, H., 2001. Temporal profile of anti-ganglioside antibodies and their relation to clinical parameters and treatment in Guillain–Barré syndrome. J. Neurol. Sci. 190, 41–47.

Ravetch, J.V., 2002. A full complement of receptors in immune complex diseases. J. Clin. Invest. 110, 1759–1761.

Romero-Steiner, S., Musher, D.M., Cetron, M.S., Pais, L.B., Groover, J.E., Fiore, A.E., Plikaytis, B.D., Carlone, G.M., 1999. Reduction in functional antibody activity against *Streptococcus pneumoniae* in vaccinated elderly individuals highly correlates with decreased IgG antibody avidity. Clin. Infect. Dis. 29, 281–288.

Sheikh, K.A., Zhang, G., Gong, Y., Schnaar, R.L., Griffin, J.W., 2004. An anti-ganglioside antibody-secreting hybridoma induces neuropathy in mice. Ann. Neurol. 56, 228–239.

Susuki, K., Nishimoto, Y., Yamada, M., Baba, M., Ueda, S., Hirata, K., Yuki, N., 2003. Acute motor axonal neuropathy rabbit model: immune attack on nerve root axons. Ann. Neurol. 54, 383–388.

Susuki, K., Nishimoto, Y., Koga, M., Nagashima, T., Mori, I., Hirata, K., Yuki, N., 2004. Various immunization protocols for an acute motor axonal neuropathy rabbit model compared. Neurosci. Lett. 368, 63–67.

Susuki, K., Rasband, M.N., Tohyama, K., Hirata, K., Baba, H., Yuki, N., 2006. Anti-GM1 IgG antibodies mediate complement-dependent destruction of sodium channel clusters in peripheral motor nerve fibers. J. Neuroimmunol. 178 (Suppl. 1), 223–224 (Abstr.).

van Sorge, N.M., van den Berg, L.H., Geleijns, K., van Strijp, J.A., Jacobs, B.C., van Doorn, P.A., Wokke, J.H., van de Winkel, J.G., Leusen, J.H., van der Pol, W.L., 2003. Anti-GM1 IgG antibodies induce leukocyte effector functions via Fcgamma receptors. Ann. Neurol. 53, 570–579.

van Sorge, N.M., van der Pol, W.L., Jansen, M.D., Geleijns, K.P., Kalmijn, S., Hughes, R.A., Rees, J.H., Pritchard, J., Vedeler, C.A., Myhr, K.M., Shaw, C., van Schaik, I.N., Wokke, J.H., van Doorn, P.A., Jacobs, B.C., van de Winkel, J.G., van den Berg, L.H., 2005. Severity of Guillain-Barré syndrome is associated with Fcgamma Receptor III polymorphisms. J. Neuroimmunol. 162, 157–164.

van Spriel, A.B., Leusen, J.H., van Egmond, M., Dijkman, H.B., Assmann, K.J., Mayadas, T.N., van de Winkel, J.G., 2001. Mac-1 (CD11b/CD18) is essential for Fc receptor-mediated neutrophil cytotoxicity and immunologic synapse formation. Blood 97, 2478–2486.

Yamaguchi, A., Katsuyama, K., Nagahama, K., Takai, T., Aoki, I., Yamanaka, S., 2004. Possible role of autoantibodies in the pathophysiology of GM2 gangliosidoses. J. Clin. Invest. 113, 200–208.

Yuki, N., Yamada, M., Koga, M., Odaka, M., Susuki, K., Tagawa, Y., Ueda, S., Kasama, T., Ohnishi, A., Hayashi, S., Takahashi, H., Kamijo, M., Hirata, K., 2001. Animal model of axonal Guillain–Barré syndrome induced by sensitization with GM1 ganglioside. Ann. Neurol. 49, 712–720.

Yuki, N., Susuki, K., Koga, M., Nishimoto, Y., Kamijo, M., 2002. Myelin-derived glycolipid and animal models of Guillain–Barré syndrome: reply. Ann. Neurol. 51, 532–533.

Zhang, G., Lopez, P.H., Li, C.Y., Mehta, N.R., Griffin, J.W., Schnaar, R.L., Sheikh, K.A., 2004. Anti-ganglioside antibody-mediated neuronal cytotoxicity and its protection by intravenous immunoglobulin: implications for immune neuropathies. Brain 127, 1085–1100.