Relation of clinical, serological, morphological, and electrophysiological findings in galactocerebroside-induced experimental allergic neuritis

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SUMMARY Rabbits were immunised repeatedly with bovine brain galactocerebroside. Almost all animals developed overt polyradiculoneuropathy. Circulating IgG antibodies to galactocerebroside in the serum and deposits of IgG in the spinal roots were detectable weeks before definite clinical, morphological, and electrophysiological alterations occurred. The levels of IgG antibody titres to galactocerebroside did not correlate with the severity of the clinical disease and of nerve conduction slowing. Remyelination and a virtually complete recovery of nerve dysfunction occurred although circulating antibodies to galactocerebroside were still present.

A chronic demyelinating disease of the peripheral nervous system can be induced in rabbits by sensitisation with galactocerebroside, a glycolipid hapten common in central and peripheral nervous system myelin.^{1,2} Clinical signs of galactocerebroside induced experimental allergic neuritis (galactocerebroside-EAN) reportedly occur two to eleven months after the initial inoculation.

Antibodies to galactocerebroside are thought to be an important pathogenic factor in this disorder. Antisera to galactocerebroside demyelinate CNS cultures, inhibit myelination in immature CNS cultures,³ and intraneural injection into rat sciatic nerves produces an acute block of nerve conduction with subsequent paranodal demyelination.^{4,5} Galactocerebroside-EAN has been proposed as a model disease for human chronic polyradiculoneuropathy where successful treatment with plasma exchange has led to the concept of pathogenic humoral factors.^{6,7}

In order to elucidate further the pathogenic role of anti-galactocerebroside *in vivo* we have examined the development of early and late clinical and electro-

physiological signs and of structural changes in relation to circulating antibody titres in this model disease. Our study indicates that there is no close relationship between antibody titres and severity of experimental allergic neuritis. Highest individual titres of circulating antibody to galactocerebroside precede the clinical disease, and remissions with remyelination of axons regularly occur despite the persistence of low titres of anti-galactocerebroside antibody.

Materials and methods

Immunisation schedule, antibody titre measurements, and clinical score

Thirty six outbred New Zealand albino rabbits (Ivanovas, Kisslegg, FRG) with 3-3.5 kg body weight were used. Twenty five were immunised with bovine brain galactocerebroside (Type 1; Sigma), 11 served as controls. Each animal received intramuscular injections of 2 ml of an inoculum in four sites on the back every 3 weeks for the first 6 months, and every 6 weeks thereafter. The inoculum contained 2 mg of galactocerebroside mixed with 10 mg methylated bovine serum albumin (mBSA, Sigma) as carrier protein in 1 ml 0.2 M phosphate buffered saline (pH 7.4) using sonification in ice water as described by Saida *et al.*¹ One ml of this solution was emulsified with 1 ml of complete Freund's adjuvant. Control animals received an inoculum containing mBSA without galactocerebroside. Prior to the first immunisation and 2 weeks after each booster injection

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serum was collected. Anti-galactocerebroside antibody titres in the IgG fraction of rabbit serum were measured by a radioimmunoprecipitation assay employing ^3H cholesterol as a marker in the galactocerebroside-containing liposomes according to the method of Fry *et al.*⁸ The dilution at which 50% of the liposomes were precipitated was taken as anti-galactocerebroside antibody titre.

Animals were scored clinically for galactocerebroside-EAN on the following scale: (+) mild, (++) moderate, and (+++) severe tremor of the hind limbs; (+) mild, (++) marked weakness, and (+++) paralysis for the evaluation of muscle strength.

Morphology and immunocytochemistry

In order to study early changes 10 of the 25 galactocerebroside immunised rabbits and five of the 11 control animals were examined morphologically 2–12 weeks after primary immunisation. Furthermore three galactocerebroside immunised and two control animals were killed 20 weeks after primary immunisation, when first electrophysiological changes could just be seen. Five galactocerebroside rabbits were examined at the chronic stage of the disease at weeks 27, 40, 59, and 60, when clinical disease was severe, likewise two control animals at week 70. The remaining animals (seven galactocerebroside-immunized, two controls) were scored for clinical signs and kept for electrophysiological measurements up to 18 months after primary immunisation without morphological examination. All animals were anaesthetised with ketamine (50 mg/kg Ketanest[®], Parke-Davis) and pentobarbital (30 mg/kg), and perfused through the left cardiac ventricle and, in addition, through the abdominal aorta with a 10% dextran solution containing 0.1 ml/500 ml of a heparin solution followed by a 2.5% phosphate buffered glutaraldehyde solution. Spinal cord with adjacent roots and peripheral nerves were dissected and fixed for an additional 2 hours. Specimens were postfixed in 2% osmium tetroxide for 3 hours and embedded in Epon. One μm sections were cut at different levels of the spinal cord, the cauda equina, and the sciatic nerves in both galactocerebroside and mBSA-immunised rabbits and stained with toluidine blue. Ultrathin sections were post-stained with uranyl acetate and lead citrate and examined on a Hitachi EM 600.

For immunocytochemical studies sciatic nerves were dissected prior to perfusion and fixed by immersion in a 4% phosphate buffered paraformaldehyde solution for 10 hours and embedded in paraffin. In addition, two galactocerebroside-immunised rabbits (4 and 70 weeks after primary immunisation) and one untreated control animal were perfused with a 4% paraformaldehyde/0.5% glutaraldehyde solution and embedded in paraffin. Ten μm sections of sciatic nerve and spinal cord with adjacent roots were mounted on slides and deparaffinised. Immunoglobulin deposits were localised by the PAP-technique⁹ as follows: after incubation with 5% bovine serum albumin in 0.01 M PBS for 30 min, goat anti-rabbit IgG antiserum (Dako) was added at dilutions 1:500 up to 1:10000 for 16 hours at 4°C, followed by incubation with sheep anti-goat IgG antiserum (Dako) 1:100 for 10 min and goat PAP-complex (Dako) 1:50 for additional 10 min at room temperature. After a rinse in 0.05 M tris-HCl buffer (pH 7.6) the peroxidase reaction was

performed with diaminobenzidine (Sigma, 10 mg/50 ml) and 0.004% H_2O_2 for 10 min.

Electroneurography

Rabbits were anaesthetised by intramuscular injections of Hypnorm[®] (Janssen, Neuss, FRG) containing 10 mg/ml fluanisone and 0.315 mg/ml fentanyl-dihydrogencitrate (0.5 ml/kg body weight). In previous experiments it was confirmed that this neuroleptic-analgesic combination did not affect peripheral and central nerve conduction. Temperature was measured by a surface thermistor at the thigh and maintained at 35–36°C by a warming lamp. Electrophysiological measurements were performed blindly with a Medelec M91a electromyograph. Stimulation was performed by 0.7 mm thick DISA needle electrodes (13L60) inserted at the sciatic notch and 2 cm proximal to the heel at the left hind leg. Evoked compound muscle action potentials (CMAP) were recorded by steel needle electrodes placed at the plantar muscle 6 cm distal to the heel (recording electrode) and at the metacarpo-phalangeal joint. A near-nerve position of the stimulating electrodes was accepted when the threshold strength for eliciting a CMAP response was 30–40 mV. Stimulation was performed with square wave pulses of 0.05 ms duration at 50% supramaximal strength. It was always ensured that the stimulus giving the shortest latency to the recorded response was taken to reduce bias. Proximal and distal amplitudes of CMAPs and distal F-wave latency were estimated and the motor nerve conduction velocity (NCV) was calculated from the obtained latencies. Mixed afferent potentials were obtained after averaging at least twenty responses at the sciatic notch with stimulation at the malleolus. The identical electrode positions as for motor conduction studies were used. All NCVs were calculated using length measurements by a caliper. During the entire experimental period testing was done with almost identical needle positions by using bone and skin marks. Somatosensory evoked potentials (SSEP) were recorded after 100–200 stimuli at the sciatic notch by DISA needle electrodes with a pseudounipolar electrode between two spinal processes at D12/L1 and with the inactive electrode placed at the lateral abdominal wall. Baseline values were obtained for each animal by measurements prior to immunisation. Ten galactocerebroside-immunised rabbits and five control animals were examined every two weeks up to the date of death at weeks 2, 4, 6, 8, and 12 after primary immunisation, others (three galactocerebroside, two controls) every 4 weeks up to week 20. Twelve galactocerebroside and four control animals were examined at various times at the chronic stage of the disease, 5 months up to 18 months after primary immunisation.

Statistical calculations were performed with the standard *t* test for grouped data.¹⁰

Results

Clinical observations and antibody titres

As the first clinical sign a mild tremor appeared 6 to 8 weeks after primary immunisation in nine out of 12 galactocerebroside-immunised rabbits observed for at least 6 months. At 4 months 10 of the 12 animals exhibited a moderate tremor (+ +). Muscle weakness

Fig 1 Representative time course of circulating IgG antibody titres to galactocerebroside and of clinical signs in a galactocerebroside-immunised rabbit. Highest individual antibody titre preceded the onset of clinical experimental allergic neuritis, and the disease persisted despite low levels of antibodies to galactocerebroside at the chronic stage of the disease. In this rabbit as a first obvious sign of experimental allergic neuritis a moderate tremor occurred at week 18. Immunisations with galactocerebroside are marked by vertical bars.

(+) usually started 4 to 5 months after primary immunisation and became marked (++) 5 to 9 months after primary immunisation. In three rabbits paralysis was present at 4 and 6 months, respectively. A typical example is shown in fig 1. Only two of the 12 animals never showed clinical signs.

Five rabbits with overt galactocerebroside-EAN were perfused at the peak of the disease, seven were kept up to 18 months. In the latter animals clinical signs remitted to only mild residual weakness within a few months despite further booster injections. All control animals, including the four rabbits observed up to 18 months, never exhibited definite clinical signs.

As early as two weeks after primary immunisation IgG-antibodies to galactocerebroside could be detected in 12 out of 15 galactocerebroside-immunised rabbits with titres ranging from 1:30 to 1:100. Five weeks after primary immunisation all animals had detectable IgG antibodies to galactocerebroside. Individual peak antibody titres ranging from 1:70 to 1:1000 were exhibited for a total of about 7 weeks. Thereafter there was a plateau of antibody titres (1:60–1:400) in 13 out of 15 galactocerebroside-immunised rabbits for about four months before titres decreased to 1:50 over the following months despite further booster injections (cf fig 1). Only two galactocerebroside-immunised animals exhibited persisting high titre antibody to galactocerebroside up to one year after primary immunisation (table 1, no 6).

The levels of antibody titres to galactocerebroside did not correlate with the severity of the clinical disease. Representative examples are shown in table 1. Two of the three paralysed rabbits exhibited high antibody titres to galactocerebroside, the other had moderately elevated antibody titres (table 1, no 5). Two animals with marked weakness exhibited antibodies to galactocerebroside with only low titres (table 1, no 3), while, in contrast, two clinically unaffected rabbits had moderately elevated antibody titres (table 1, no 4). In sera obtained from mBSA-immunised control rabbits and in pre-immune sera of galactocerebroside immunised animals antibodies to galactocerebroside could never be detected.

Morphology and immunocytochemistry

Early after immunisation with galactocerebroside, that is up to 10 weeks, no pathological changes were seen. Some vacuolar disarrangement and splitting of myelin sheaths were the earliest morphological signs

Table 1 Relation between clinical signs of experimental allergic neuritis and antibody titres to galactocerebroside in representative animals

Animal no	Time after primary immunisation			
	5 weeks	14 weeks	6 months	8 months
1	586* +/0	282 ++/+	232 +++/++	100 +++/+
2	214 0/0	208 ++/0	122 +++/++	36 +++/++
3	70 +/0	60 +/0	57 +/+	50 +/++
4†	856 0/0	208 0/0	70 0/0	— 0/0
5‡	872 0/0	256 +/+	70 ++/++	† —
6	920 0/0	384 +/0	768 ++/+	200 +++/++

*The reciprocals of the antibody titres are listed. Below each titre on the left the clinical grade for tremor and on the right the grade for weakness is given as described in *Materials and methods*.

†Killed 7 months after primary immunisation.

‡Note the nearly identical antibody titre courses of rabbits no 4 and 5 in contrast to the striking differences in clinical signs.

Fig 2 Dorsal spinal root of a galactocerebroside-immunised rabbit at week 20 after primary immunisation when electrophysiological measurements revealed disperse and delayed SSEPs. Numerous axons close to the venule (asterisk) are completely demyelinated (triangle). Mononuclear phagocytes are crowded around the vessel. The arrow indicates a fibre with myelin splitting (1 μ m plastic section) $\times 330$

in the spinal roots and peripheral nerves 12 weeks after primary immunisation.

The galactocerebroside-immunised rabbits examined at week 20 after primary immunisation showed perivenular demyelination of fibres predominantly in the dorsal spinal roots. Some myelin sheaths were split, others were undergoing active demyelination (fig 2). Phagocytosing mononuclear cells were fre-

Fig 3 Electronmicrograph showing a mononuclear phagocyte which is loaded with myelin debris and encircles a demyelinated axon enveloped by its Schwann cell cytoplasm $\times 7400$

Fig 4 Electronmicrograph showing thinly myelinated fibres at the dorsal spinal root of a galactocerebroside-immunised rabbit 9 months after primary immunisation. ($\times 3300$)

quently seen in these lesions. Electron microscopic investigations established these cells as macrophages sometimes enveloping denuded axons (fig 3). Large fibres with an abnormally thin myelin sheath indicating remyelination were only occasionally seen. In the more distal parts of the peripheral nerves the only abnormal finding was occasional myelin splitting.

At the peak of the clinical disease (weeks 27, 40, 59, 60) marked and widespread demyelination with many denuded fibres were seen in the ventral and dorsal spinal roots. In some instances axons with denuded internodes followed by unaffected or thinly myelinated internodes could be traced on longitudinal plastic sections indicating segmental demyelination. Thinly myelinated fibres, surrounded by a basal membrane as revealed by electron microscopy, were frequently seen in these animals (fig 4). In the peripheral nerves paranodal demyelination was found predominantly around venules, but these changes were always much less pronounced than in the spinal roots.

Lymphocytic infiltrates were never observed in our animals at any stage of the disease. The spinal cord was always spared. Control rabbits did not exhibit pathological changes even 18 months after primary immunisation despite repeated injections with mBSA and CFA.

Peripheral nerves and spinal roots of galactocerebroside-immunised rabbits at the peak of the disease immunocytochemically stained for IgG deposits always showed intense reaction products at the endoneurial space at a 1:10000 dilution of goat anti-rabbit IgG antiserum (fig 5). In rabbits before the onset of clinical experimental allergic neuritis and in

Fig 5 Immunocytochemical localisation of IgG in the sciatic nerve of a paretic galactocerebroside rabbit 9 months after primary immunisation. (1 µm plastic section). The dark reaction product is deposited at the endoneurial space between nerve fibres and in the lumina of two capillaries (arrows). Goat anti-rabbit IgG antiserum was used at a dilution of 1:2000. The poor fixation of the myelin sheaths is due to fixation by immersion with glutaraldehyde without osmication to preserve the antigenicity of the IgG. Inset: immunocytochemical localisation of IgG in the sciatic nerve of a normal rabbit (10 µm paraffin section, goat anti-rabbit IgG 1:2000). Only a faint staining occurs at the endoneurial space. (Nomarski-optics) (× 330)

controls a faint staining was seen at the endoneurial space at a 1:2000 dilution of primary antibodies (fig 5, inset). Replacement of primary antibodies by PBS or by goat anti-mouse IgG antiserum (1:2000) abolished all immunoreactivity.

Electroneurography

Sequential measurements of galactocerebroside-immunised rabbits 2, 4, 6, 8, and 12 weeks after pri-

Fig 6 Lumbar somato-sensory evoked potentials of a galactocerebroside-immunised rabbit prior to immunisation, 4 and 10 months after primary immunisation. For stimulation and recording see text. Latencies are prolonged, and potentials are disperse and decreased amplitude (Note change in gain and time scale)

Fig 7 Representative time course of changes in nerve conduction in a galactocerebroside-immunised rabbit. Compound muscle action potentials evoked after stimulation at the sciatic notch (proximal) and at the malleolus (distal) and recorded at the plantar muscle. Note the parallel decrease of CMAP amplitudes after proximal and distal stimulation at months 4 and 10, and the prolonged CMAP and F-wave latencies. At month 18 nerve conduction clearly improved reaching nearly pre-immunisation values.

mary immunisation showed no changes in peripheral nerve conduction, including lumbar SSEPs. As a first electrophysiological sign latencies of SSEPs were prolonged, and potentials became more dispersed with a decrease in amplitudes, usually around 4 months after primary immunisation (fig 6). Within the following 5 months motor and mixed afferent NCVs decreased, and F-wave and distal latencies became prolonged. Proximally and distally evoked CMAP amplitudes gradually decreased in roughly the same way indicating that at most only a minor fraction of the fall in amplitude was due to conduction block between stimulation sites (fig 7). The most prominent pathological changes were seen 9 months after primary immunisation (table 2). Starting at about 12 months after primary immunisation all abnormal findings slowly improved reaching nearly pre-experimental values within the following months. Only one animal still exhibited pathological SSEPs 16 months after primary immunisation.

In mBSA-immunised control animals nerve conduction always remained unchanged. Baseline values of the galactocerebroside-immunised rabbits were not different from control values.

Discussion

In this paper we confirm previous investigations indicating that repeated immunisation of rabbits with

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Table 2 Statistical evaluation of the electrophysiological changes in galactocerebroside immunised rabbits 9 months after primary immunisation compared with pre-immunisation values

	9 months* (n = 11)	Pre-experimental values (n = 15)
Motor NCV (m/s)	48 ± 5.7	69.4 ± 7.7
Distal latency (ms)	3.6 ± 1.0	1.9 ± 0.1
F-wave latency (ms)	14.9 ± 2.8	10.1 ± 0.3
Mixed afferent NCV (m/s)	67.3 ± 9.3	95 ± 8.3
Distally evoked CMAP amplitudes (mV)†	4.1 ± 1.5	12.1 ± 2.8
Proximally evoked CMAP amplitudes (mV)†	3.2 ± 1.1	8.7 ± 2.7

*Means ± standard deviation all values significant at $p < 0.001$.

†Peak to peak measurements of the main component.

Note that there is no additional reduction of compound muscle action potential (CMAP) amplitudes between proximal and distal stimulation sites.

NCV: nerve conduction velocity.

galactocerebroside induces a chronic demyelinating polyradiculoneuropathy which resembles human chronic Guillain-Barré syndrome in some aspects.¹² In extension of these studies we now have demonstrated that circulating antibodies to galactocerebroside precede the onset of clinical and electrophysiological signs of experimental allergic neuritis, and progressively fall thereafter associated with clinical remission despite further sensitisation with the hapten. In individual animals and between different rabbits antibody titres were not closely linked to the signs of disease.

The severity of clinical disease correlated in time and degree with morphological and electrophysiological changes in individual animals. At the onset of the disease the dorsal spinal roots were predominantly affected as indicated by structural changes and abnormal SSEPs. During the chronic stage of the disease both efferent and afferent fibres were involved. A conduction block at more distal sites with the electrophysiological sign of a fall in amplitude between proximal and distal stimulation sites was not a common feature in our animals, in contrast to findings in experimental allergic neuritis,¹¹ human Guillain-Barré syndrome,¹² and to the initial report on galactocerebroside-induced EAN.¹ Slowing of nerve conduction and increased dispersion of the evoked muscle responses were similar to other experimental demyelinating neuropathies such as diphtheritic neuropathy,¹³ lysophosphatidylcholine-induced neuropathy,¹⁴ and some cases of classical experimental allergic neuritis with little or no axonal damage,^{15,16} but the basic difference from these conditions is the slow time course of the nerve disorder in galactocerebroside-EAN.

Some observations deserve further interpretation in this model disease. It is surprising that thinly remyelinated axons were frequently seen at the chronic stage of experimental allergic neuritis where

antibody titres to galactocerebroside were still elevated. Moreover, remyelinating fibres could occasionally be detected even in an early phase of ongoing demyelination. Ultimately, a virtually complete clinical recovery followed despite the persistence of circulating antibodies to galactocerebroside. This contrasts with other experimental observations demonstrating that galactocerebroside antibodies inhibit myelination *in vitro*,³ and probably prevent remyelination in the Theiler virus encephalomyelitis.¹⁷ It is conceivable that newly formed peripheral myelin is protected by some unknown mechanisms although the putative antigen, galactocerebroside, is expressed even early in myelination.¹⁸

The invariably slow development of segmental demyelination in galactocerebroside-immunised rabbits is difficult to understand in view of the reported fulminant effects after intraneural transfer of rabbit sera containing anti-galactocerebroside antibodies.^{5,19} It has been postulated that the delayed onset of galactocerebroside-EAN is due to the blood nerve barrier preventing a rapid access of circulating antibodies to their target.² Our sequential immunocytochemical observations do not support this view, because we could demonstrate polyclonal IgG deposits in the spinal roots and peripheral nerves long before structural or electrophysiological alterations were seen. This is, in accordance with previous studies showing that the blood nerve barrier is not tight at the spinal root level.²⁰ Although we did not specifically demonstrate anti-galactocerebroside antibodies it seems unlikely that these were excluded from passing through a broken barrier.

Other, as yet undefined factors are likely to be critical for rabbit galactocerebroside-EAN hyperimmune sera to exert either acute or chronic demyelinating effects or to allow for remyelination in an otherwise ongoing demyelinating neuropathy.

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