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Experimental Allergic Neuritis Induced by Sensitization with Galactocerebroside

Abstract. *Thirteen of 31 rabbits immunized repeatedly with bovine brain galactocerebroside developed experimental allergic neuritis, manifested by flaccid paresis and hypesthesia of four limbs, 2 to 11 months after the initial inoculation. Electrophysiological studies revealed multifocal conduction block of peripheral nerves. Perivenular demyelinative lesions associated with phagocytic mononuclear cells occurred in spinal ganglia, roots, and less frequently in distal nerves.*

Experimental allergic neuritis (EAN) and experimental allergic encephalomyelitis (EAE) are autoimmune, demyelinating diseases of the peripheral nervous system (PNS) and central nervous system (CNS); they are classically produced in animals by injection of homogenates of PNS or CNS tissue, respectively, with complete Freund's adjuvant (*1*). Experimental allergic neuritis can be induced by injection of P2 basic protein (BP) of peripheral nerve myelin. A peptide from myelin P2 BP is at least one of the neuritogenic determinants (*2*). In EAE, the encephalitogen is CNS myelin BP (*3*, *4*). Another major component of CNS and PNS myelin (*5*), galactocerebroside (β-D-galactopyranosyl ceramide) (GC), is a glycolipid hapten (*6*, *7*) and binds specific antibodies in isolated myelin (*7–9*). Antiserum to GC binds GC in both central and peripheral myelin in sections of rat optic and sciatic nerve, as well as in CNS myelin of unfixed spinal cord cultures as demonstrated by indirect immunofluorescence or immunoperoxidase techniques (*10*). Galactocerebroside can serve as a cell-surface antigenic marker for oligodendrocytes in culture (*10*). Further, rabbit antiserum to GC demyelinates organotypic CNS cultures and inhibits myelination and sulfatide synthesis in immature CNS cultures (*11–13*). However, immunization with one or two injections of GC has not been encephalitogenic (*6*). We describe here the first successful production of EAN in rabbits by repeated immunization with GC. The distribution of demyelinative lesions seems to correspond to areas known to have a defective blood-nerve barrier (*14*, *15*).

Thirty-one male New Zealand albino rabbits, weighing 2.3 to 2.7 kg, were sensitized with GC up to seven or eight times following one of three schedules (I to III) (Table 1). The immunizing inoculum contained 1 or 2 mg of bovine brain GC (lower spot cerebrosides, 98 percent with hydroxylated fatty acids) (Sigma) and bovine serum albumin or egg albumin (Sigma) as a carrier protein (5 mg per milligram of GC), in complete Freund's adjuvant (Difco) or, for booster injections in schedules II and III, without adjuvant. Galactocerebroside was checked for purity by thin-layer chromatography on silica gel G plate developed with chloroform, methanol, and water (65:25:4, by volume) using six standard sphingolipids and phosphoglycerides (*16*). Two spots were obtained. The major spot (> 99 percent) had an R_F of 0.85 and the minor spot (< 1 percent) had an R_F of 0.88, corresponding, respectively, to cerebrosides with longer (24 carbon) or shorter (16 or 18 carbon) length fatty acids. Analysis by thin-layer and gas chromatography after hydrolysis revealed that galactose was the only carbohydrate moiety detectable (> 99.9 percent). Sixteen control rabbits were similarly immunized but without GC. Since results with the three schedules did not vary significantly, we will describe the clinical, pathological, and serological results as grouped data.

Thirteen of 31 rabbits immunized with GC developed a neurological disorder, with onset ranging from day 44 to day 314 (135 ± 21 days, mean ± standard error) after the initial inoculation (Table 1). Rabbits were maintained for a maximum of 1 year. Subacute onset of weight loss, tremulousness, ataxia, flaccid paresis, and hypesthesia of four limbs were the main features of the clinical illness (Fig. 1A). Progress was sometimes rapid; quadriplegia and respiratory paresis were terminal events in three animals less than 2 weeks after onset of signs of illness. None of the control rabbits showed neurological abnormalities.

Animals immunized following schedule I were subjected to electrophysiological studies prior to terminal histological examination. The characteristic abnormality was multifocal conduction block (Fig. 1, E and F). In animals examined from 2 to 24 weeks after onset of weakness, motor conduction velocities were diffusely slowed (11 m/sec; normal is 50 m/sec), suggesting widespread peripheral nerve demyelination. These electrophysiological abnormalities were indistinguishable from those found in human multifocal demyelinative neuropathies such as Guillain-Barre syndrome (*17*).

Twenty-three GC-immunized rabbits, including all 13 paralyzed rabbits at various clinical stages, and 12 control rabbits were killed at corresponding intervals between 1 month to 1 year after immuni-

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zation and studied pathologically (*18*). All rabbits with clinical weakness had multifocal demyelinative lesions, seen with light and electron microscopy, primarily in spinal ganglia, roots, cauda equina, and less frequently in peripheral nerves (Fig. 1, B and C). Demyelination started around venules, with splitting and vacuolation of the outer myelin sheaths of nearby fibers, and later formed confluent lesions. The lesions were associated with infiltration of phagocytic mononuclear cells, mostly macrophages, which insinuated themselves between myelin lamellae, phagocytized the myelin sheath, and denuded the axon (Fig. 1D). In contrast to the EAN or EAE produced by sensitization with

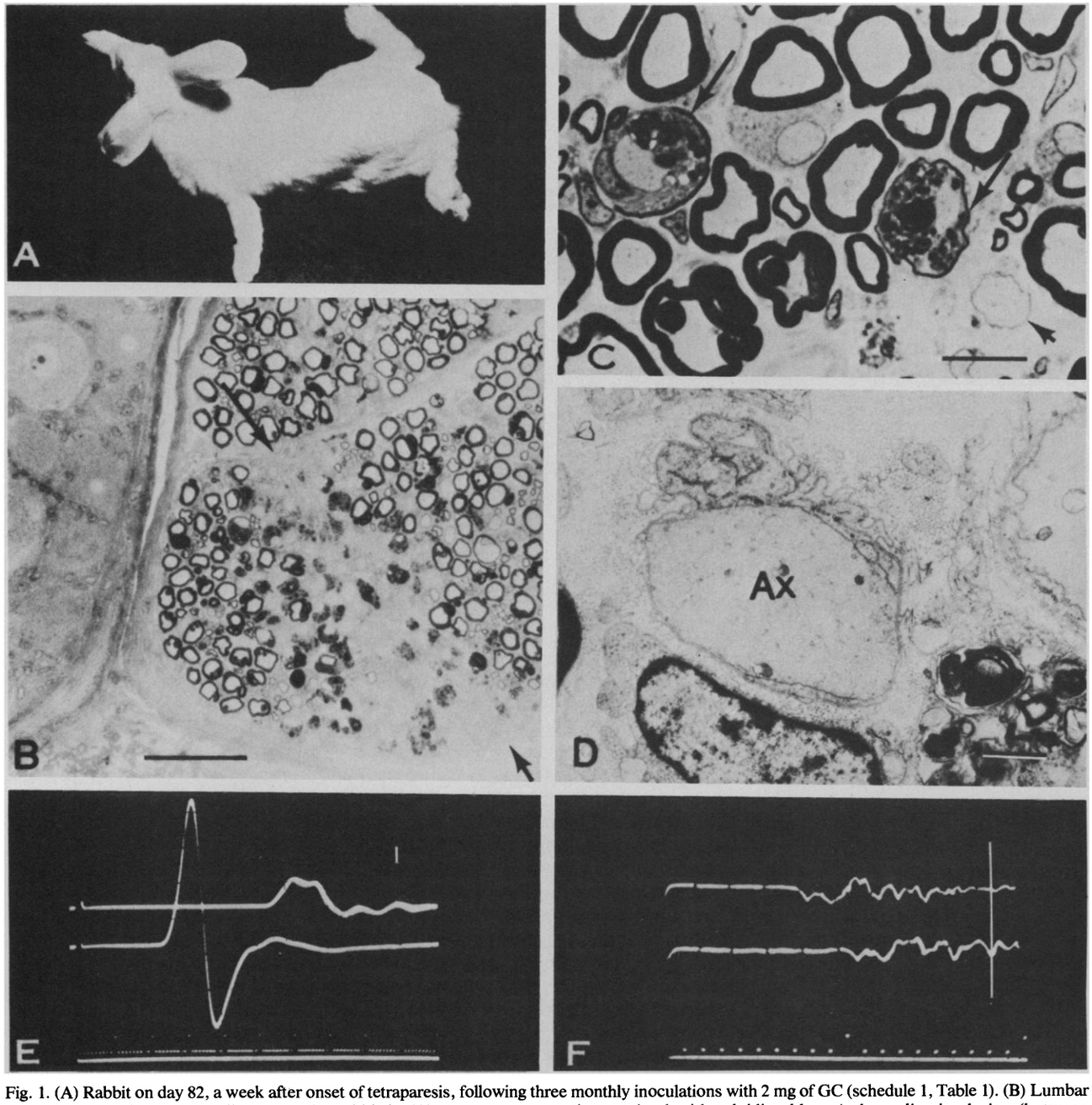

Fig. 1. (A) Rabbit on day 82, a week after onset of tetraparesis, following three monthly inoculations with 2 mg of GC (schedule 1, Table 1). (B) Lumbar ventral root and spinal ganglion from the rabbit in (A), 1-μm epoxy sections stained with toluidine blue. A demyelinative lesion (between arrows) consists of denuded axons and lipid-laden macrophages. The bar represents 100 μm. (C) Higher-magnification view of the root in (B) showing active demyelination. Processes of macrophages have insinuated underneath the myelin and contain phagocytized myelin debris (arrows). An axon with a thinly remyelinated sheath is also present (arrowhead). The bar represents 20 μm. (D) Electron micrograph of (B) showing a denuded axon (*Ax*) and a macrophage with myelin figures in its cytoplasm. This ultrathin section is stained with uranyl acetate and lead citrate. The bar represents 2 μm. (E) Sciatic nerve motor conduction study of a clinically affected rabbit 4 days after the onset of mild paresis, showing partial conduction block between hip and ankle. Plantar muscle responses were recorded with subcutaneous needle electrodes. Upper trace: supramaximal sciatic nerve stimulation at sciatic notch. Lower trace, supramaximal posterior tibial nerve stimulation at ankle. Distance, 143 mm; calibration, 1 mV; time signal, 0.1 and 1 msec. Although conduction in the fastest motor fiber is normal (53 m/sec) the response evoked by hip stimulation is of low amplitude and dispersed. The ankle response amplitude and distal motor latency are normal. (F) Sciatic nerve motor conduction study in an animal with mild paresis and ataxia of 7 days' duration, showing marked prolongation of terminal motor conduction. Upper trace; hip stimulation. Lower trace, ankle stimulation. Distance, 160 mm; calibration, 1 mV; time signal, 1 and 10 msec. Note that although sciatic nerve conduction from hip to ankle is normal (53.5 m/sec), distal motor latency is extremely prolonged (7.6 msec; normal $\leq$ 2.5 msec), and the muscle responses are dispersed and of low amplitude.

whole PNS or CNS tissue (*1*), or with neuritogenic or encephalitogenic BP or peptide (*2, 4*), or to Guillain-Barre syndrome (*19*), perivenular infiltration of small lymphocytes was not found. Minimal demyelinative lesions in spinal roots and ganglia were also found in six of ten clinically normal GC-immunized rabbits. The root entry zone, area postrema, and all other parts of the CNS studied (*18*) were normal. Thus, demyelinative ganglioradiculoneuritis was produced experimentally by immunizing with GC. The degree of morphological changes correlated with clinical and electrophysiologic abnormalities. None of 12 autopsied control rabbits showed abnormalities in the PNS or CNS.

The production of serum antibody to GC was monitored periodically (*20*) by the agglutination of liposomes containing GC (*21*). All GC-immunized rabbits showed various degrees of elevation of antibody to GC to a titer $\geq$1:32 within 3 weeks (Table 2) (all preimmunization serums were $\leq$1:8). The repetitive immunization did not give significantly higher titers. The highest titers during the course of each GC-immunized rabbit with clinical illness (1:32 to 1:1024, median and mode = 1:128) tended to be higher than that of the clinically normal GC-immunized group (1:32 to 1:512, median = 1:64, mode = 1:32). However, four rabbits with high titers of antibody to GC ($\geq$1:128) did not show any clinical abnormality.

Antibody titers to glucocerebroside, lactocerebroside, mixed brain gangliosides type III (Sigma), ceramide, sphingosine, sphingomyelin (Applied Science) as well as GC were measured by a radioimmunoprecipitation test employing [^{3}H]cholesterol as a marker in micelles containing the appropriate lipid hapten (*21*). There was no significant elevation of titer to these lipids other than GC. Antibody to GC was not absorbed significantly when high titer antiserums to GC were incubated with an ethanolic suspension of each of the above lipids other than GC or monogalactosyl diglycerides (Applied Science), together with cholesterol and lecithin (*22*). These results indicate the specificity and cross-reactivity of these antiserums to GC, and also the importance of a galactose moiety for haptenic activity of GC (*6, 7, 22, 23*).

To rule out possible CNS-BP contamination in the immunizing inoculum containing bovine brain GC preparation, antigen binding capacity of rabbit serum to CNS-BP was determined by coprecipitation of ^{125}I-labeled antigen-antibody complexes (*24*). All antiserums to GC obtained either before or after clinical onset, or serums from control rabbits, bound at the preimmunization serums level of 1 to 4 percent. Four hyperimmune rabbit antiserums to CNS-BP tested at the same time bound 10, 13, 24, or 36 percent.

Rabbit antiserum to GC demyelinates not only cultured CNS (*8, 11, 12*) and PNS tissue in vitro (*8*), but also peripheral nerve in vivo (*9, 25*) after the direct subperineurial injection of antiserum (*18, 26*). The demyelinating factor in antiserum to GC seems to be antibody (*8, 9, 12*). In normal animals of some species including the rabbit, the blood-nerve barrier is defective at the dorsal root ganglion and nerve root, and less frequently at the distal peripheral nerve (*14, 15*). This could provide an access route for circulating demyelinating antibodies to GC. This distribution corresponds well to that of the demyelinative lesions in GC-immunized rabbits. The reported variation between individual animals of the same species in the leakiness of serum

Table 1. Incidence of EAN in rabbits immunized with GC plus albumin (GC) and albumin alone (Control). The three schedules of GC immunization were as follows. (I) Injected intramuscularly at four sites on the back with 2 ml of inoculum monthly for the first four times, then every 3 months thereafter, and kept up to 1 year; the inoculum contained 2 mg of GC, 10 mg of bovine serum albumin (BSA), 1 ml of complete Freund's adjuvant (CFA) with 5 mg of heat-killed, dried *Mycobacterium tuberculosis* H37Ra (Difco), and 1 ml of phosphate buffered saline (PBS) (*p*H 7.4). (II) Injected intramuscularly with 1 ml of inoculum every 2 weeks for the initial three times, and intraperitoneally as boosters every 3 to 4 weeks thereafter, and kept up to 7 months; the initial inoculum contained 2 mg of GC, 10 mg of BSA, 0.5 ml of CFA, and 0.5 ml of PBS. For booster inoculum additional PBS was substituted for CFA. (III) Injected with 0.5 ml of inoculum, containing 1 mg of GC, 5 mg of egg albumin, 0.25 ml of CFA, and 0.25 ml of PBS, intradermally at four foot pads for the first injection and weekly for the three successive intramuscular injections. Booster injections were given thereafter intraperitoneally six times, until day 130 after the first sensitization, with the same inoculum except that additional PBS was substituted for CFA. Control rabbits of each group were inoculated by the same protocol used for GC sensitization except that GC was omitted from the inoculum.

Diagnosis	Immunization schedule					
	I		II		IIII	
	GC (N = 19)	Control (N = 8)	GC (N = 5)	Control (N = 3)	GC (N = 7)	Control (N = 5)
Clinical						
EAN	9*	0	2*	0	2*	0
Normal	10	8	3	3	5	5
Histological						
EAN	13	0	3	0	3	0
Normal	2	8	1	3	1	1

*All rabbits with clinical EAN were autopsied and found to have histological evidence of EAN.

Table 2. Relationship of the titer of antibody to GC to clinical EAN in four representative rabbits immunized with GC (following schedule III in Table 1). Antibody was titrated by a GC-liposome agglutination assay. Rabbit No. 1 developed clinical EAN on day 56 when the titer of antibody to GC reached a high level. Rabbit No. 2 was normal when titer became very high for the first time at day 28, but developed EAN 2 months later at the second peak of antibody titers. Animal No. 3 responded to GC only weakly and remained normal. Rabbit No. 4 showed no EAN signs despite high level of titers that continued for several months.

Day of	Day of antiserum assay	Antibody titer of rabbits with			
		Clinical EAN		No clinical EAN	
		No. 1	No. 2	No. 3	No. 4
Inoculation					
0	0	1:2	1:2	1:4	
7	7			1:2	
14	14	1:8			
21	21	1:64	1:256		1:32
	28	1:32	1:1024	1:32	
Booster injection					
50	56	1:256*	1:256	1:32	1:256
	63				
76	84		1:512*	1:32	1:256
86	94		1:1024*		1:256
	101		1:512*		1:256
108	108				1:128
	116		1:256*		1:128

*Clinical EAN present.

protein into the nerve parenchyma (*15*) may account for the relative independence of the disease induction from the level of antibody titer. Initially after immunization with GC, antibodies to GC consist predominantly of immunoglobulin (Ig) M. Immunoglobulin G against GC may not rise for several months (*27*). Although IgG is able to permeate into the endoneurium, IgM, a larger molecule than IgG, cannot penetrate the nonpathological nerve (*28*). It is possible that the delayed appearance of IgG against GC coincided with the induction of the demyelinative neuritis. Although GC reportedly does not induce positive skin reactions (*6*), the possible role of cell-mediated immunity cannot be excluded. However, the absence of perivenular cuffings of small lymphocytes in the early lesions in this study also supports the concept that GC-induced EAN may be primarily antibody-dependent rather than the result of tuberculin-type delayed hypersensitivity.

Possible involvement of GC in the immunopathogenesis of EAE and EAN produced by whole white matter or nerve homogenates, has been implied by the detection of antibody to GC in the serum of such animals (*6-8, 12, 29*). Antibody to GC was also found in cerebrospinal fluid of EAE animals (*30*). The present report provides evidence that GC is capable of playing a primary role in the production of a demyelinating disorder.

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GABA Receptor Control of Parasympathetic Outflow to Heart: Characterization and Brainstem Localization

Abstract. *Blockade of γ-aminobutyric acid (GABA) receptor function by direct microinjection of the GABA receptor antagonist bicuculline into the nucleus ambiguus of the brainstem produced a marked, dose-related depression of heart rate and blood pressure which was mediated by the vagus nerve. This effect was not obtained in other regions of the brainstem and was reversed by the GABA receptor agonist muscimol. These data indicate that the nucleus ambiguus may be the site of a GABA receptor-mediated inhibition of vagal outflow.*

The recent availability of muscimol, a potent and selective agonist for γ-aminobutyric acid (GABA) receptors (*1*) has spurred investigations into the role of GABA receptors in specific brain regions for the control of such diverse functions as food intake (*2*), motor activity (*3*), synthesis of various neurotransmitters (*4*), and release of pituitary hormones (*5*). Moreover, the combined use of muscimol and the specific GABA receptor antagonist bicuculline (*1*), has allowed the exploration of the effects of both augmentation and reduction in GABA receptor function in various brain systems. Recently, evidence has emerged suggesting that GABA receptors might also be involved in the central autonomic control of cardiovascular function (*6, 7*), but the role and anatomical site (or sites) of such a GABA influence have not been characterized. Antonaccio and co-workers have reported that muscimol, administered into the cerebral ventricles, decreased heart rate and blood pressure and that this effect of muscimol was reversed by the GABA antagonists bicuculline and picrotoxin (*6*). In contrast, we have reported that bicuculline and picrotoxin, administered via the verte-

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