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Systemic complement depletion reduces inflammation and demyelination in adoptive transfer experimental allergic neuritis

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Abstract The effect of systemic complement depletion by cobra venom factor (CVF) was evaluated in adoptive transfer experimental allergic neuritis (AT-EAN). Spleen cells of rats immunized with a neuritogenic peptide SP26 were injected into naive rats. On days 3 and 6 after cell transfer AT-EAN rats were treated with CVF or saline intraperitoneally. AT-EAN rats treated with CVF had significantly lower scores for histological inflammation (0.25 ± 0.25 vs 1.9 ± 0.4, mean ± SEM, $P < 0.03$) and demyelination (0.13 ± 0.13 vs 1.6 ± 1.4, $P < 0.02$) than saline-treated AT-EAN rats. Immunocytochemistry of lumbosacral nerve roots showed significantly less ED1-positive macrophages (0.5 ± 0.3 vs 1.6 ± 0.6, $P < 0.04$) and CD11bc-positive (expressing complement receptor 3 or CR3) inflammatory cells (0.6 ± 0.4 vs 1.7 ± 0.5, $P < 0.03$). Our data suggest that complement plays a crucial role in inflammatory demyelination since systemic complement depletion significantly reduces recruitment of macrophages into the nerve and subsequent macrophage-mediated demyelination.

Key words Adoptive transfer experimental allergic neuritis · Complement · Macrophage · Inflammation · Demyelination

Introduction

Guillain-Barré syndrome (GBS) is an acute inflammatory demyelinating polyradiculoneuropathy in which an immune response following an infection is directed against components of peripheral nerve. Evidence of humoral factors in the pathogenesis of GBS is illustrated by the favorable response to plasmapheresis, the presence of serum complement fixing anti-peripheral nerve myelin (anti-PNM) antibodies and of complement activation products in serum and cerebrospinal fluid (CSF) [15, 17, 26]. Detailed autopsy studies in demyelinating GBS have shown early complement fragment C3d deposition on the Schwann cell membrane and vesicular myelin changes before macrophages invade the nerves and strip off myelin lamellae [8]. In the more recently recognized axonal form of GBS, complement and IgG are deposited on the ad-axonal membrane and macrophages are found later in the axonal space [7]. PNM can activate complement through the alternative pathway in vitro and acute-phase GBS serum causes complement-dependent demyelination in culture and after intraneural application [16, 20, 27].

Experimental allergic neuritis (EAN), the in vivo model of GBS, is produced in Lewis rats by immunization with PNM or its neuritogenic components and is characterized by inflammatory demyelination of the peripheral nerves leading to tail and limb weakness. Transfer of antigen-specific T cells produces a similar disorder with a shorter latency called adoptive transfer EAN (AT-EAN), suggesting that EAN is a T cell-mediated disease [23]. However, there is evidence that in PNM-induced EAN humoral factors, including complement and antibodies to myelin antigens, enhance the peripheral nerve damage induced by T cells [1, 14, 33]. Intraneural injection of EAN serum and complement or intraperitoneal (i.p.) injection of EAN serum in conjunction with opening of the blood-nerve barrier results in peripheral nerve demyelination [9, 12, 13, 22, 25].

In EAN the membrane attack complex of complement, C5b-9, is deposited early on myelin and Schwann cells before there is significant cellular infiltration [28]. Complement depletion by cobra venom factor (CVF) reduces the severity of clinical disease and histological demyelination in PNM-induced EAN [5]. We demonstrated that in addition, complement depletion causes a significant re-

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duction in the number of inflammatory cells infiltrating the nerve roots [32]. This effect of CVF on clinical and histopathological disease severity is abrogated if more severe EAN is induced with a high dose of PNM.

In the current study we investigate the effect of systemic complement depletion with CVF in AT-EAN.

Materials and methods

Immunization and cell transfer

Female Lewis rats (Charles River), 160–180 g in weight, were immunized with 300 mg SP26 (a neuritogenic peptide comprising amino acids 53–78 of the bovine P2 protein [24], synthesized in the Department of Analytical Chemistry at the University of Texas) emulsified in an equal volume of complete Freund's adjuvant (CFA) which contained 10 mg *Mycobacterium tuberculosis* per ml. The immunogen was injected in 200 ml into the footpads of each hindleg. Rats were killed on day 13. Spleens were removed and spleen cells were incubated at 2×10^6 cells/ml with 2.5 mg/ml SP26 in RPMI (Gibco, Grand Island, N. Y.) supplemented with 10% fetal calf serum (FCS; Whittaker Bioproducts, Walkersville, Md.), 1 mM sodium pyruvate (Gibco), 2 mM glutamine (Gibco), 100 units penicillin/streptomycin and 50 μM 2-mercaptoethanol. The plates were incubated at 37° C for 2 days in a humidified atmosphere containing 5% CO_2. Cultured spleen cells were then resuspended and injected i. v. in naive Lewis rats at 50×10^6 cells per animal for adoptive transfer of EAN. Data from two separate experiments are presented.

Clinical assessment

The rats were weighed daily and evaluated clinically using the following scale: 0, normal; 1, limp tail; 2, abnormal gait; 3, mild paraparesis [10].

Complement depletion

On days 3 and 6 after adoptive transfer of SP26-specific spleen cells rats were injected with CVF (Sigma, St. Louis, Mo.; 0.7 ml i. p.) or saline. This dose of CVF was found to deplete systemic complement to a CH_{50} (total hemolytic complement activity) of less than 5% of control samples in previous experiments [32].

Histology

Rats were killed on day 9 after adoptive transfer. The cauda equina was removed and divided. The distal portion was fixed in 2.5% paraformaldehyde/2.5% glutaraldehyde in phosphate-buffered saline (PBS) for 4–6 h, osmicated overnight in 2% osmium tetroxide, dehydrated and embedded in Epon. Three to four blocks of the lower lumbosacral roots were prepared from each animal. Representative 0.5-μm sections of each block were stained with toluidine blue; per animal 50–60 roots or rootlets were evaluated.

Histological assessment of demyelination and inflammation was performed using the following scales [10]. For demyelination: 1+, a few demyelinated axons perivenular or scattered; 2+, many foci of perivenular demyelination; 3+, extensive demyelination perivenular and confluent; for inflammation: 1+, a few scattered mononuclear inflammatory cells often subperineurial; 2+, perivenular cuffing with mononuclear inflammatory cells; 3+, extensive multifocal perivenular cuffing and widespread endoneurial inflammation. All slides were read by an investigator (M. P.), who was unaware of treatment protocol pertaining to the specimens.

The proximal portion of the cauda equina (± 2 cm) was snap frozen in methylbutane cooled in liquid nitrogen and stored at –70° C. Cross-sections (8 μm thick) were cut on a cryostat, fixed in ice-cold acetone and used for immunocytochemistry.

Immunocytochemistry

Immunocytochemistry was performed using the following antibodies: ED1 1 : 1000, ED2 1 : 50, ED3 1 : 50 (macrophages), W3/13 (lymphocytes) 1 : 50, C3 1 : 1000 (from Cappel, Calif.), CD11b/c 1 : 100 (CR3 on macrophages, granulocytes, dendritic cells; PharMingen, Calif.). Frozen sections (8 μm thick) were blocked in normal rabbit serum for 30 min, then incubated with the primary antibody for 1 h, washed with PBS, incubated with biotinylated secondary antibody for 1 h, washed in PBS, incubated with avidin-biotin-peroxidase complex (ABC; Vectastain) for 45 min, washed and developed with diaminobenzidine and hydrogen peroxide. Blocking with 0.01% hydrogen-peroxide preceded incubation with primary antibodies to C3.

The intensity and distribution of staining in affected nerve roots was scored as: 1+, mild; 2+, moderate; and 3+, severe; by an investigator (M. P.) blinded to the source of the specimen.

Results

Clinical disease

The AT-EAN rats developed only minor weight loss that was not significantly different between CVF-treated and saline-treated rats (CVF treated 5.8 ± 4.6 g; saline treated 4.7 ± 2.4 g). Clinical signs of weakness were not discernible in CVF or saline-treated AT-EAN rats.

Immunocytochemistry

CD11bc-positive imflammatory cells and ED1-positive macrophages were found both clustered perivascularly and more diffusely infiltrating affected nerve roots. The number of cells expressing CD11bc was usually slightly higher than the number of ED1-positive macrophages in areas of inflammation.

In AT-EAN rats treated with CVF significantly fewer ED1-positive macrophages and CD11bc-expressing inflammatory cells were present in lumbosacral nerve roots compared to saline-treated AT-EAN rats (Figs. 1, 2). C3 staining paralleled the severity of the inflammatory infiltrate and C3 deposition was invariably present where inflammation received a score of 2–3. In all but one of the animals treated with CVF no C3 deposition was seen. W3/13-positive lymphocytes were found in areas of inflammation, but there were consistently fewer W3/13-positive cells than ED1-positive macrophages (W3/13 score for CFV-treated rats: 0.22 ± 0.34; for saline treated rats 1.2 ± 1.3).

No significant difference was observed between the CVF-treated and control group in staining for ED2, a marker of resident macrophages. ED2-positive cells were seen sporadically in most nerves but did not specifically cluster in areas of inflammation. ED3-positive macro-

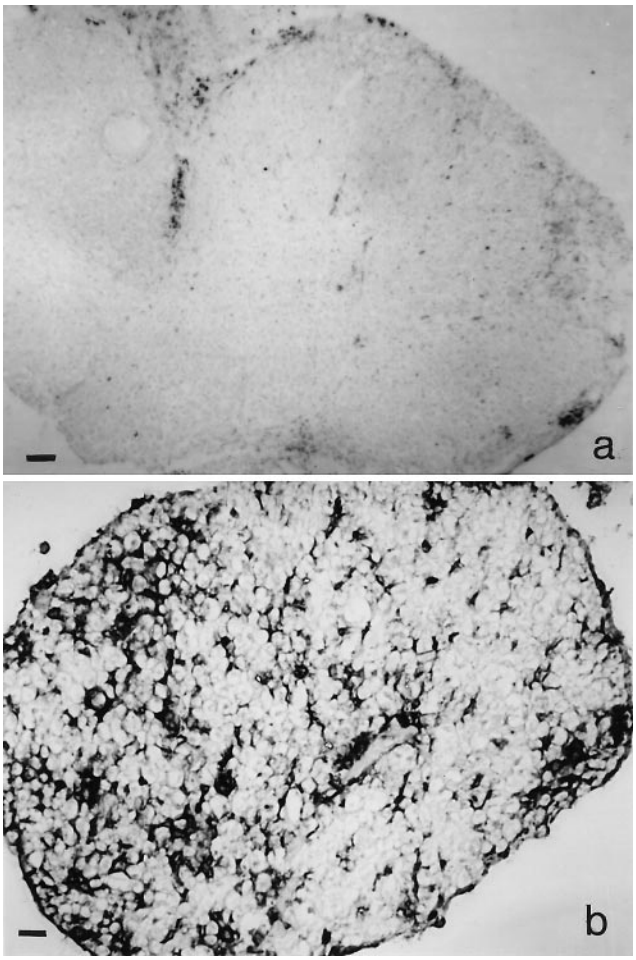

Fig. 1 Immunocytochemical localization of macrophages, granulocytes and dendritic cells expressing CR3 using CD11bc antibody in lumbosacral nerve roots of CVF-treated **(a)** and saline-treated **(b)** rats with AT-EAN. CVF-treated animals show significantly fewer CD11bc-positive cells than saline-treated animals (*CR3* complement receptor 3, *CVF* cobra venom factor, *AT-EAN* adoptive transfer experimental allergic neuritis). *Bar* 20 μm

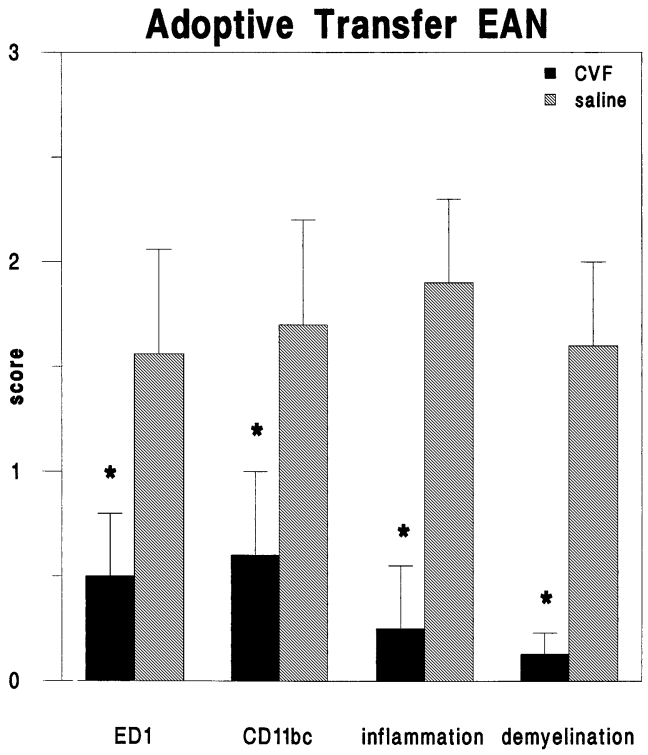

Fig. 2 Lewis rats with AT-EAN were treated with CVF ($n = 8$) or saline ($n = 8$) on day 3 and 6 after cell transfer. Immunocytochemistry of lumbosacral nerve roots obtained on day 9 showed significantly fewer ED1-positive macrophages and CD11bc-positive cells (macrophages, granulocytes and dendritic cells expressing CR3) in CVF-treated animals compared to saline-treated controls (ED1: 0.5 ± 0.3 vs 1.6 ± 0.6, mean ± SED $P < 0.04$; CD11bc: 0.6 ± 0.4 vs 1.7 ± 0.5 $P < 0.03$). Histological scores for inflammation and demyelination were significantly reduced after CVF treatment (inflammation: 0.25 ± 0.25 vs 1.9 ± 0.4, mean ± SEM $P < 0.02$; demyelination: 0.13 ± 0.13 vs 1.6 ± 0.4, $P < 0.03$, Wilcoxon)

phages were found only in areas of inflammation with large collections of CD11bc-positive cells, but they were fewer in number than the ED1-positive macrophages.

Histology

Epon-embedded sections of lumbosacral nerve roots of AT-EAN rats showed the characteristic abnormalities of inflammatory demyelination with both perivascular inflammation and more diffusely infiltrating inflammatory cells. Large foamy macrophages containing myelin debris were observed. Perivascular- and macrophage-associated demyelination was present. Both inflammation and demyelination were strikingly reduced in CVF-treated compared to saline-treated animals (Fig. 3). In only one of eight CVF-treated rats were inflammatory cells and demyelinated fibers found, while in the other seven animals histological sections were completely normal. In saline-treated AT-EAN rats, completely normal histology was seen in only one animal.

Discussion

The results of our experiments show that depletion of systemic complement with CVF significantly reduces both inflammation and demyelination of peripheral nerves in AT-EAN. Complement deposition is decreased concomitantly with inflammation. Overall the disease is mild and remains subclinical both in control and CVF-treated AT-EAN animals.

The current data in the antibody-independent model of AT-EAN are analogous to the previously reported results for EAN induced by low doses of myelin (CVF vs saline-treated EAN rats: ED1: 0.4 ± 0.2 vs 1.6 ± 0.3, $P < 0.01$; CD11bc: 1 ± 0.3 vs 2.5 ± 0.3, $P < 0.01$; inflammation: 0.6 ± 0.4 vs 2 ± 0.4, $P < 0.02$; demyelination: 0.4 ± 0.3 vs 1.9 ± 0.3, mean ± SEM, $P < 0.01$, Wilcoxon) [32], suggesting a similar influence on the effector phase of the disease.

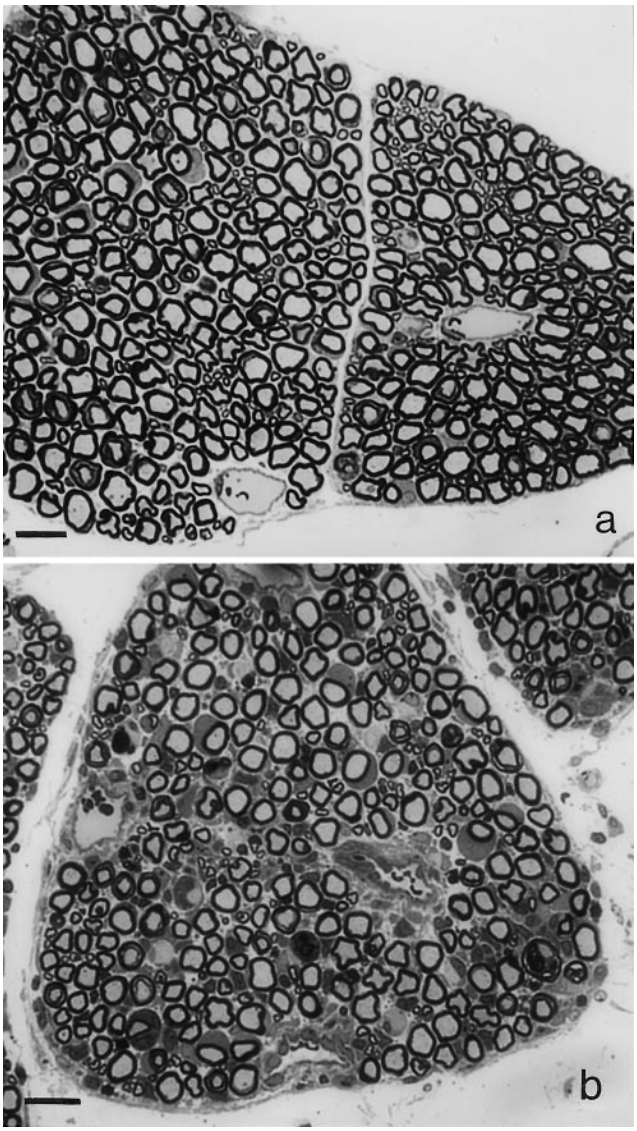

Fig. 3 Epon-embedded sections of the cauda equina of rats with AT-EAN show no or minimal perivascular inflammation and demyelination in CVF-treated animals (**a**) compared to saline-treated rats (**b**). *Bar* 10 μm

The induction phase with T cell activation for AT-EAN takes place in the donor animal, whereas in myelin-induced EAN CVF treatment is started on day 9 post immunization, after disease induction. The effector phase of AT-EAN entails migration of activated T cells across the blood-nerve barrier, recruitment of macrophages into the endoneurium and macrophage-mediated demyelination. Following the initial rolling of leukocytes mediated by selectins, chemotactic factors such as C5a or cytokines such as interferon-γ and tumor necrosis factor-α (TNF-α) induce endothelial cell activation, which in turn activates adhesion molecules on the leukocytes. Cell migration is accomplished through interactions between integrins on migrating cells and adhesion molecules on endothelial cells [4, 29].

CVF, a form of Cobra 3b, binds factor B to form a C3 convertase CVFBb, which systemically cleaves C3, thereby releasing large amounts of C3a and C3b fragments since it is not subject to regulatory factor H and I. Systemic C3 depletion follows this initial short-lasting massive complement activation [31]. Systemic complement depletion with CVF may affect endoneurial recruitment of inflammatory cells in several ways, by inhibiting the formation of C5a, which is a strong chemoattractant, or by diminishing ICAM-1 up-regulation on endothelial cells by TNF-α since this is influenced by complement activation and C5b-9 deposition [30].

The reduction of demyelination seen in our CVF-treated EAN rats may be simply secondary to the reduced recruitment of macrophages into the nerve. However, complement is also necessary to opsonize myelin for phagocytosis by macrophages [2, 6]. C3 fragment deposition can occur by antibody-dependent or -independent mechanisms. Although in GBS circulating specific antibodies together with complement contribute to demyelination [15], the activation of complement in vitro by PNM via the alternative pathway supports the fact that antibodies may not be necessary for complement-mediated demyelination [16].

Our study suggests that in AT-EAN the predominant effect of systemic complement depletion by CVF is on adherence and recruitment of inflammatory cells into the nerve. Initial influx of complement components from the circulation may be affected, which will reduce opsonization and the effectiveness of macrophage-mediated demyelination. However, since macrophages, Schwann cells and fibroblasts have the capability to synthesize complement components [3, 20], once sufficient inflammatory cells enter the endoneurial compartment demyelination can proceed. This may explain why the effect of CVF treatment was mostly abrogated when immunization was performed with high-dose myelin which causes a more severe clinical and histological disease.

Other investigators recently published comparable results in abstract form using a T cell line to induce EAN [11]. Several studies have focused on the role of complement in experimental allergic encephalomyelitis (EAE) [18, 19, 21]. In EAE, produced by immunization with myelin basic protein or by adoptive transfer of a low dose of antigen-specific spleen or T cells, systemic complement depletion with CVF reduced clinical disease and early histological inflammation. The demyelination that occurs in these EAE models after additional injection with anti-myelin oligodendrocyte glycoprotein (anti-MOG) antibodies also was diminished if complement was depleted prior to antibody injection. In more severe disease complement depletion had no significant effect. The authors concluded that the major role of the complement cascade in EAE appeared to be the generation of proinflammatory factors, and that in the absence of complement components an antibody-dependent cell-mediated cytotoxic response may play a role in the pathogenesis of antibody-mediated demyelination [21]. Our findings in EAN are comparable to those in EAE. In summary, the findings of

dramatically decreased peripheral nerve inflammation and demyelination after systemic complement depletion in the AT-EAN model support a crucial role for complement in the effector phase of immune-mediated inflammatory demyelination. Treatments that reduce complement activation may be beneficial in human inflammatory demyelinating polyneuropathy.

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References

1. Archelos JJ, Roggenbuck K, Schneider-Schaulies J, Toyka KV, Hartung HP (1993) Detection and quantification of antibodies to the extracellular domain of P0 during experimental allergic neuritis. J Neurol Sci 117:197–205
2. Bruck W, Friede RL (1990) Anti-macrophage CR3 antibody myelin phagocytosis by macrophages in vitro. Acta Neuropathol 80:415–418
3. Dashill S, Vanguri P, Koski CL (1994) Cytokines and cyclic AMP induce C3 production in rat Schwann cells. J Immunol 150:225A
4. Dianzani U, Malavasi F (1995) Lymphocyte adhesion to endothelium. Crit Rev Immunol 15:167–200
5. Feasby TE, Gilbert JJ, Hahn AF, Neilson M (1987) Complement depletion suppresses Lewis rat experimental allergic neuritis. Brain Res 419:97–103
6. Griffin JW, Lobato GR, Tyor WR, Li CY, Glass JD (1992) Macrophage responses and myelin clearance during Wallerian degeneration: relevance to immuno-mediated demyelination. J Neuroimmunol 40:153–166
7. Hafer-Macko CE, Hsieh ST, Li CY, Ho TW, Sheikh K, Cornblath DR, McKhann GM, Asbury AK, Griffin JW (1996) Acute motor axonal neuropathy: an antibody-mediated attack on axolemma. Ann Neurol 40:635–644
8. Hafer-Macko CE, Sheikh KA, Li CY, Ho TW, Cornblath DR, McKhann GM, Asbury AK, Griffin JW (1996) Immune attack on the Schwann cell surface in acute inflammatory demyelinating polyneuropathy. Ann Neurol 39:625–635
9. Hahn AF, Gilbert JJ, Feasby TE (1980) Passive transfer of demyelination by experimental allergic neuritis serum. Acta Neuropathol (Berl) 49:169–176
10. Hahn AF, Feasby TE, Wilkie L, Lovgren D (1991) P_2-peptide induced experimental allergic neuritis: a model to study axonal degeneration. Acta Neuropathol 82:60–65
11. Hahn AF, Lovgren D, Wilkie L, Nicolle M (1996) The role of complement in the pathogenesis of inflammatory demyelination. Neurology 46:A397
12. Harvey GK, Pollard JD (1992) Peripheral nervous system demyelination from systemic transfer of experimental neuritis serum. J Neuroimmunol 41:159–166
13. Harvey GK, Gold R, Hartung HP, Toyka KV (1995) Non-neural-specific T lymphocytes can orchestrate inflammatory peripheral neuropathy. Brain 118:1263–1272
14. Koehler NKU, Martin R, Wiethölter H (1996) The antibody repertoire in experimental allergic neuritis: evidence for PMP-22 as a novel neuritogen. J Neuroimmunol 71:179–189
15. Koski CL, Humphrey R, Shin ML (1985) Anti-peripheral myelin antibody in patients with demyelinating neuropathy: quantitative and kinetic determination of serum antibody by complement component 1 fixation. Proc Natl Acad Sci USA 82:905–910
16. Koski CL, Vanguri P, Shin ML (1985) Activation of the alternative pathway of complement by human peripheral nerve myelin. J Immunol 134:1810–1814
17. Koski CL, Sanders ME, Swoveland PT (1987) Activation of terminal components of complement in patients with Guillain-Barré syndrome and other demyelinating neuropathies. J Clin Invest 80:1492–1497
18. Linington C, Lasmann H, Morgan BP, Compston DAS (1989) Immunohistochemical localisation of terminal complement component C9 in experimental allergic encephalomyelitis. Acta Neuropathol 79:78–85
19. Linington C, Morgan BP, Scolding NJ, Wilkins P, Piddlesden S, Compston DAS (1989) The role of complement in the pathogenesis of experimental allergic encephalomyelitis. Brain 112:859–911
20. McPhaden AR, Whaley K (1993) Complement biosynthesis by mononuclear phagocytes. Immunol Res 12:213–232
21. Piddlesden S, Lassmann H, Laffafian I, Morgan BP, Linington C (1991) Antibody-mediated demyelination in experimental allergic encephalomyelitis is independent of complement membrane attack complex formation. Clin Exp Immunol 83:245–250
22. Pollard JD, Harrison B, Gatenby P (1981) Serum-induced demyelination: an electrophysiological and histological study. Clin Exp Neurol 18:70–80
23. Rostami A, Burns JB, Brown M, Rosen J, Zweiman B, Lisak RP, Pleasure DE (1985) Transfer of experimental allergic neuritis with P2-reactive cell lines. Cell Immunol 91:354–361
24. Rostami A, Gregorian SK, Brown MJ, Pleasure DE (1990) Induction of severe experimental autoimmune neuritis with a synthetic peptide corresponding to the 53–78 amino acid sequence of the myelin P2 protein. J Neuroimmunol 30:145–151
25. Saida T, Saida K, Silberberg DH, Brown MT (1978) Transfer of demyelination by intraneural injection of experimental allergic neuritis serum. Nature 272:639–641
26. Sanders ME, Koski CL, Robbins D (1986) Terminal complement pathway activation in cerebrospinal fluid in Guillain-Barré syndrome and multiple sclerosis. J Immunol 136:4456–4459
27. Sawant-Mane S, Clark MB, Koski CL (1991) In vitro demyelination by serum antibody from patients with Guillain-Barré syndrome requires terminal complement complexes. Ann Neurol 29:397–404
28. Stoll G, Schmidt B, Jander S, Toyka KV, Hartung HP (1991) Presence of the terminal complement complex (C5b-9) precedes myelin degradation in immune-mediated demyelination of the rat peripheral nervous system. Ann Neurol 30:147–155
29. Dianzani U, Malavasi F (1995) Lymphocyte adhesion to endothelium. Crit Rev Immunol 15:167–200
30. Vaporciyan AA, Mulligan MS, Warren JS, Barton PA, Miyasaka M, Ward PA (1995) Up-regulation of lung vascular ICAM-1 in rats is complement dependent. J Immunol 155:1442–1449
31. Von Zabern I (1993) Effects of venoms of different animal species on the complement system. In: Sim RB (ed) Activators and inhibitors of complement. Kluwer, Dordrecht, pp 127–131
32. Vriesendorp FJ, Flynn RE, Pappolla MA, Koski CL (1995) Complement depletion affects demyelination and inflammation in experimental allergic neuritis. J Neuroimmunol 58:157–165
33. Zhu J, Link H, Weerth, Linington C, Mix E, Qiao J (1994) The B-cell repertoire in experimental allergic neuritis involves multiple myelin proteins and GM1. J Neurol Sci 125:132–137