American Journal of Pathology, Vol. 148, No. 1, January 1996

Short Communication

Downregulation of Microglial Keratan Sulfate Proteoglycans Coincident with Lymphomonocytic Infiltration of the Rat Central Nervous System

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The monoclonal antibody (MAb) 5D4 against a keratan sulfate (KS) epitope of bovine cartilage proteoglycan stains ramified microglia in the rat brain. In this study we show that 5D4-positive microglia is abundant in the normal rat spinal cord and nearly absent during both the active and recovery phase of experimental autoimmune encephalomyelitis (EAE) in myelin-immunized Lewis rats. In contrast, during Wallerian degeneration of the optic nerve the density of KS-immunoreactive microglia remains constant. KS immunoreactivity is absent from both normal and transected sciatic nerves, and spinal nerve roots. On immunoblots of spinal cord extracts MAb 5D4 stains a novel type of KS proteoglycans (KSPGs) with an apparent molecular weight mainly between 140 and 200 kd, which significantly decrease in acute EAE. Our data suggest that high levels of KSPG expression correlate to a downregulated immunophenotype of resident macrophages in the nervous system. The lack of detectable KS in peripheral nerve points to a divergent differentiation of bone marrow-derived resident macrophages in the peripheral and central nervous systems and may partially account for the rapid macrophage response to axonal injury in the peripheral nervous system. Downregulation of microglial KSPG could be a prerequisite for a rapid inflammatory response in the central nervous system. (Am J Pathol 1996, 148:71–78)

Microglial cells constitute a population of resident central nervous system (CNS) macrophages and are considered important effector cells in the pathogenesis of human CNS diseases such as multiple sclerosis (MS), Alzheimer's disease, and HIV encephalopathy.[1–3] Most likely, the cells originate from circulating monocytes that invade the CNS early during postnatal development.[4] In the normal CNS microglial cells are characterized by a highly ramified morphology, a slow turnover rate from circulating monocytes, and a profound downregulation of several immunological macrophage functions.[5] In response to injury these cells adopt a macrophage-like morphology and increasingly express various immunomolecules including major histocompatibility complex class II antigens on their surface. This activation process occurs not only in immune-mediated CNS disease,[2,6] but to a certain extent also in nonimmune lesion paradigms such as wallerian degeneration (WD)[7] and facial nerve axotomy.[5]

Compared with microglia, the resident macrophages of the peripheral nervous system (PNS) exhibit a less extensively ramified morphology, a higher level of constitutive class II antigen expression, and a much higher rate of replacement from circulating bone-marrow derived monocytes.[8] The macrophage response during WD occurs more rapidly in the PNS than in the CNS.[9] Because phagocytic cells remove potentially nonpermissive myelin debris from the de-

Supported by the Deutsche Forschungsgemeinschaft, SFB 194 (B6).

Accepted for publication September 12, 1995.

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generating distal nerve stump[10] and secrete growth-promoting cytokines,[11] the delayed macrophage response in the CNS might partly account for its limited capacity for axonal regeneration after injury.

Recently, the monoclonal antibody (MAb) 5D4 has been used as a marker for ramified microglia in the adult rat brain.[12] The antibody was originally raised against chondroitinase ABC-digested proteoglycan (PG) monomers from bovine cartilage and recognizes an epitope in the keratan sulfate (KS) side chain of the PG molecule.[13] Because PGs are increasingly recognized as important participants in cell regulation processes,[14] we became interested in the molecular identity of the 5D4-associated PG and its role in the normal and diseased nervous system. As a first approach to this issue we studied the expression of the 5D4 epitope in immune-mediated CNS disease in comparison with WD and examined its distribution in the PNS by immunocytochemistry and immunoblot analysis. Experimental autoimmune encephalomyelitis (EAE) served as a well established model of T cell-mediated autoimmune demyelination in the Lewis rat.[6] We provide evidence that MAb 5D4 reacts with a novel type of microglial KSPG that is strongly downregulated during EAE but not WD and is absent from both normal and degenerating peripheral nerve.

Materials and Methods

Animals

Eight week-old female Lewis rats were immunized with 10 mg of bovine spinal cord myelin in complete Freund's adjuvant (CFA). Controls received CFA without CNS myelin in the same manner. In another set of experiments the left optic nerve was crushed in the orbit, whereas the left sciatic nerve was transected at the sciatic notch, both under deep anesthesia with chloralhydrate.

Immunocytochemistry

EAE animals were analyzed on day 11 (n = 4), 12 (n = 1), 13 (n = 3), 14 (n = 3), 15 (n = 5), 17 (n = 2), 18 (n = 2), 22 (n = 1), 29 (n = 2), 33 (n = 2), 34 (n = 1), 42 (n = 1), and 71 (n = 2) after immunization, those undergoing WD at weekly intervals up to 2 months after nerve injury. Animals who had their sciatic nerve transected were in addition sacrificed on days 2, 4, and 7. Animals were perfused transcardially in deep anesthesia with 4% paraformaldehyde, and tissue specimens embedded in paraffin. Five-μm sections were stained using the avidin-biotin complex peroxidase technique as previously described.[7] Primary antibodies were as follows (sources and working dilutions in parenthesis): MAb 5D4 against keratan sulfate (ICN Biomedicals, Costa Mesa, CA, 1:5000), MAb 15–6A1 against rat T cells (Holland Biotechnology, Leiden, The Netherlands, 1:500 dilution), and the macrophage marker ED1 (Serotec, Oxford, UK, 1:1000). Nonspecific labeling was checked by replacing primary antibodies with normal mouse serum (Sigma Chemical Co., Munich, Germany) at their working dilutions and was not observed.

Spinal Cord Extraction

Rats were killed with an overdose of ether. The lumbal and lower thoracic part of the spinal cord (a total of 300 mg/animal) was prepared, finely minced using razor blades, and extracted in 15 vol of ice-cold extraction buffer (0.05 mol/L sodium acetate, pH 5.8, 4 mol/L guanidine hydrochloride, 0.5% CHAPS (Sigma Chemical Co.), 10 mmol/L EDTA, with 1 μg/ml aprotinin and 100 μg/ml PEFA-Block (both from Boehringer Mannheim, Mannheim, Germany) as protease inhibitors) for 48 hours at 4°C. After centrifugation for 1 hour at 100,000 $\times g$ (4°C), the supernatant was filtered and stored at −80°C until further use.

Gel Electrophoresis

Spinal cord extract was precipitated with an equal volume of 20% trichloroacetic acid. The pellet was solubilized by boiling in sample buffer containing 2% sodium dodecyl sulfate (SDS) and 100 mmol/L dithiothreitol. Protein concentration of samples was measured using the Bio-Rad microassay (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin (BSA) standards in sample buffer. Eighty μg of total protein from both EAE and control animals were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) in 4 to 10% gradient gels. Equal loading was confirmed by silverstaining gels after blotting.

Immunoblot

Proteins were transferred electrophoretically to nitrocellulose (ECL Hybond, Amersham, Braunschweig, Germany). The membrane was blocked in 100 mmol/L Tris/Cl pH 7.5, 0.9% NaCl (TBS) containing 5% BSA/0.5% nonfat dry milk. MAb 5D4 was applied at 1:5000 dilution in TBS containing 0.5% BSA/0.5% nonfat dry milk. Bound antibody was detected using peroxidase-conjugated goat anti mouse immunoglobulin G (Jackson Immunoresearch, West Grove,

PA, 1:5000) followed by enhanced chemiluminescence (ECL detection reagents and Hyperfilm-ECL, Amersham).

Results

KS Expression in the CNS and PNS of Controls

In accordance with the study by Bertolotto et al,[12] we found expression of 5D4 immunoreactivity (IR) on a subpopulation of ramified microglia in the normal rat brain that were mainly located in the hemispheric white matter, the hippocampus, the thalamus, and more abundantly in the brain stem and cerebellum (data not shown). A significantly greater number of 5D4-reactive cells with the typical morphology of ramified microglia was distributed equally in the gray and white matter of the lumbar spinal cord (Figure 1, A and B). There was no difference between untreated animals and adjuvant controls. A lower density of KS-positive microglia was found in the normal optic nerve (Figure 3A). 5D4 IR was absent from spinal nerve roots and sciatic nerves of all the animals examined (Figure 3B).

KS Expression in Immune-Mediated Inflammation of the CNS

Under the present immunization regime ~75% of immunized rats developed moderate to severe EAE starting on day 12 post-immunization (p.i.). Around day 25 p.i. most of the diseased animals had completely recovered. Histopathologically, the most pronounced inflammation was found in the lower thoracic and lumbar spinal cord,[6] which was used for systematic immunohistological evaluation. In four immunized asymptomatic rats examined on day 11 p.i., ie, before the expected onset of clinical disease, KS IR was identical to controls (Figure 1C). None of these "presymptomatic" animals exhibited spinal cord infiltration by ED1-positive macrophages and T cells (Figure 1, E and G). By contrast, essentially all rats with the clinical picture of full-blown EAE showed extensive spinal cord infiltration by T cells and ED1-positive macrophages, and KS IR was nearly abolished (Figure 1, D, F, and H). During the recovery phase the inflammatory infiltrates were reduced, but still no reexpression of KS on microglia was detected after 1 month (n = 4) and 2 months (n = 2) p.i. (not shown).

Generally, the downregulation of KS occurred very sharply and pronounced so that on day 15, ie, 3 days after the onset of disease, essentially no KS-positive cells could be detected in the spinal cord. In some animals with mild early disease, inflammatory infiltrates were accentuated in the surrounding meninges and the subarachnoid space (Figure 2A). These animals exhibited significantly reduced but still detectable KS expression (Figure 2B). In CNS regions where inflammation was less prominent, ie, brain stem and brain, variable degrees of KS IR were still detectable even in severely affected animals and essentially inversely related to the extent of inflammatory infiltration by T cells and macrophages. This was particularly evident in the brain stem, where KS IR was abolished in foci of perivascular inflammation (Figure 2, C and D) but was still present in neighboring tissue devoid of inflammation (Figure 2, E and F).

KS Expression in WD

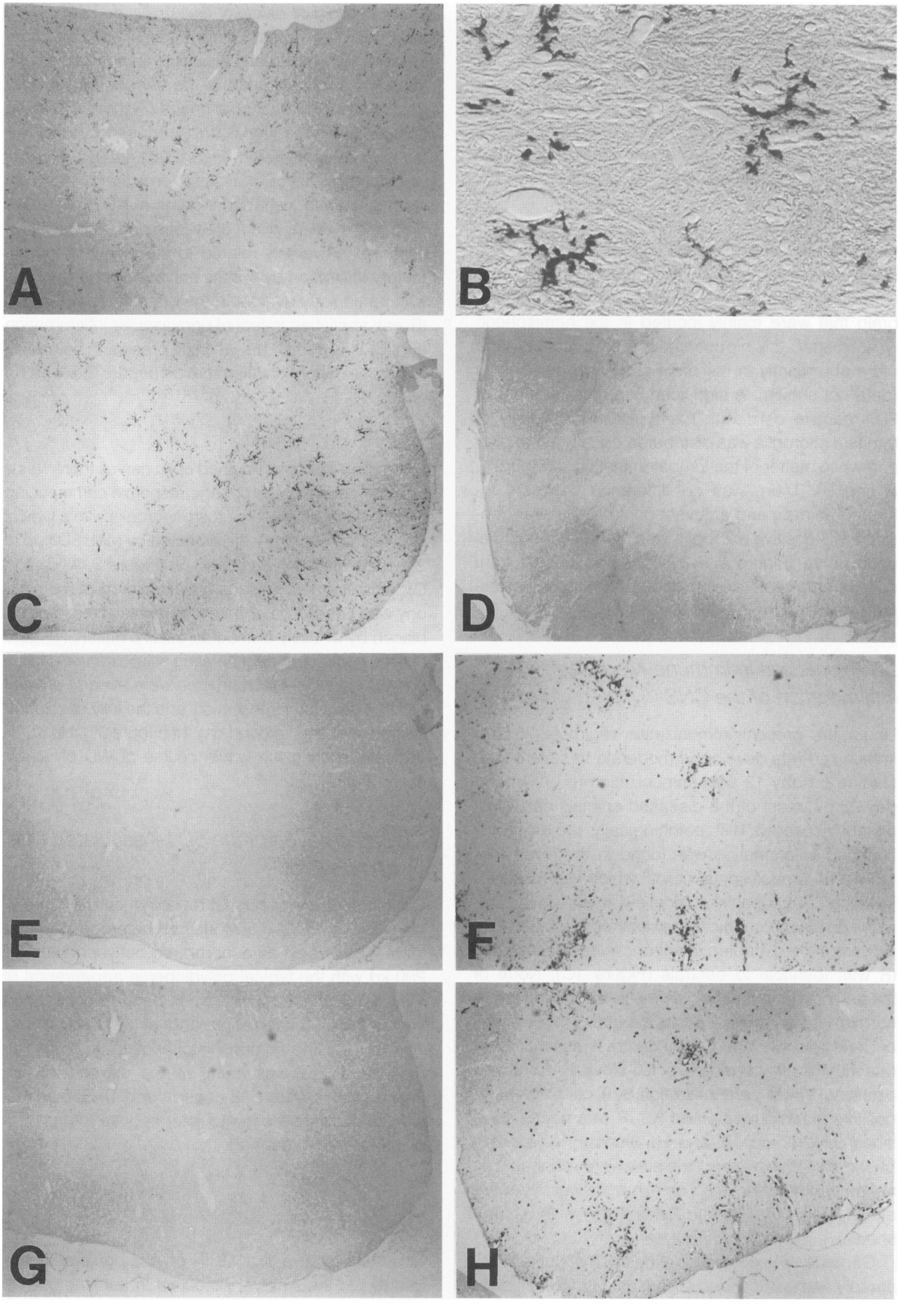
In the distal stump of crushed optic nerves there was a typically delayed macrophage response commencing around day 7 after nerve crush. Microglia with a typical ramified morphology were detected by MAb 5D4 up to 2 months after nerve transection (Figure 3, A, C, and D). At later time points the typical microglial morphology was not always apparent and was sometimes replaced by diffusely distributed dot-like material (Figure 3D). By contrast, in degenerating sciatic nerves numerous ED1-positive macrophages were already present at 3 to 4 days after transection, and the fully developed infiltrate was seen around day 14 (Figure 3F). No KS IR appeared during the whole course of WD of sciatic nerve (Figure 3E).

Characterization of the 5D4-Associated PGs by Immunoblot

On immunoblots of normal rat spinal cord (Figure 4, lanes 3 and 5) MAb 5D4 stained heterogenous material. The largest amount ranged between 140 and 200 kd with the typical polydisperse appearance of PGs. Additionally, several weak bands were stained above the 205-kd marker and at ~60 kd. Corresponding to the immunohistochemical findings, almost no KSPG was found in the extracts of two animals with severe EAE (lanes 1 and 4). One animal with mild disease showed intermediate intensity of immunostaining (lane 2).

Discussion

This study shows that KS IR on microglial cells decreases to the limits of detection in autoimmune in-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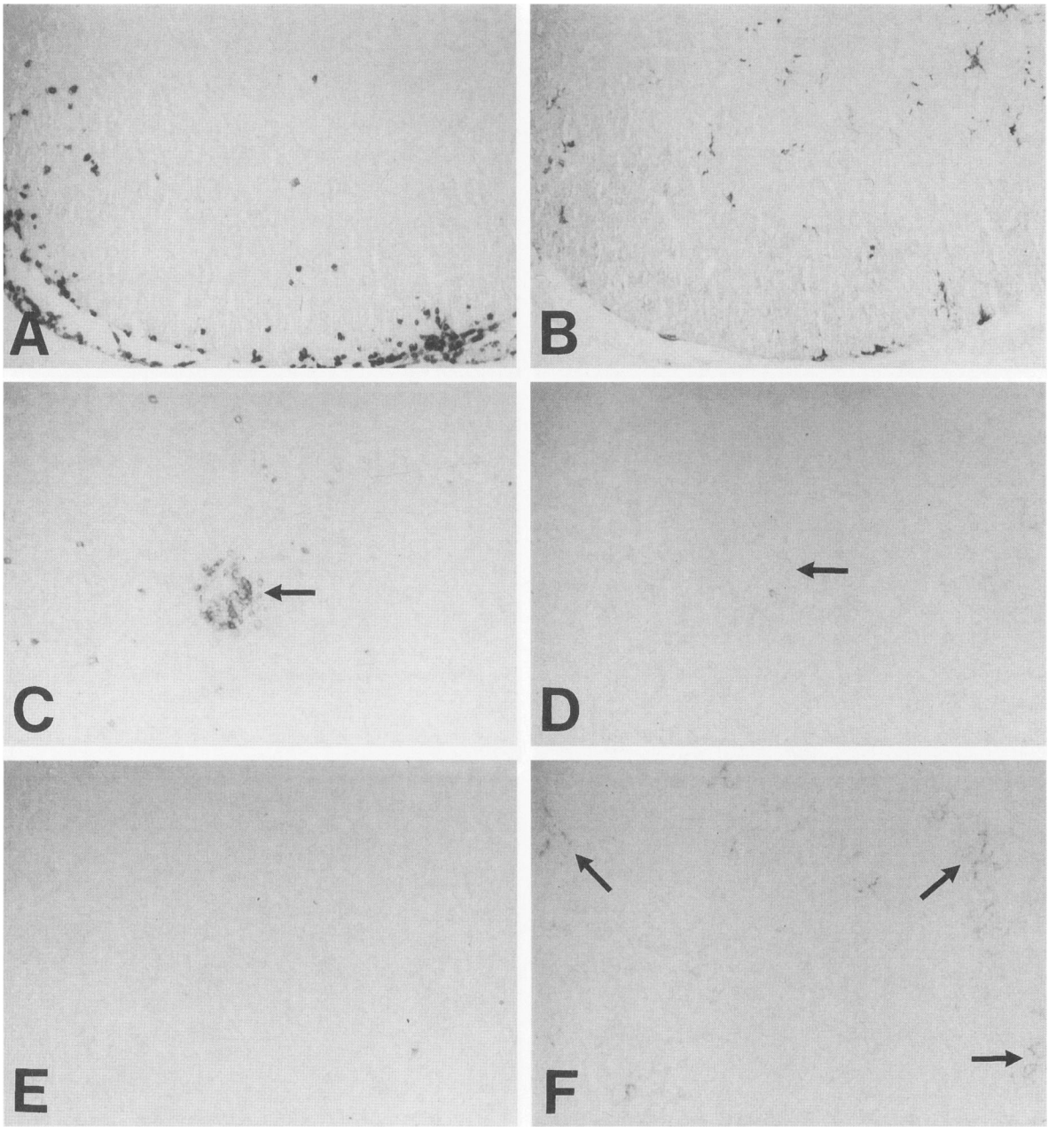

Figure 2. *KS IR in early EAE (spinal cord) and brainstem regions. T cell infiltration* (A, C, *and* E) *and KS expression* (B, D, *and* F) *in the spinal cord on day 12 p.i.* (A *and* B) *and the brainstem on day 15 p.i.* (C–F). *Note that in early EAE, T cell infiltration is accentuated in the subarachnoid space* (A). *KS expression is reduced on a serial section particularly in the immediately underlying spinal cord parenchyma, while still strong in central areas devoid of T cells* (B). *In the brainstem, foci of perivascular T cell infiltration are found in full-blown EAE* (C). *In a corresponding serial section, no KS expression is detectable* (D). *Arrows denote an identical vessel in* C *and* D. *Contrastingly, in neighboring areas devoid of T cells* (E) *KS-positive ramified microglia* (F, arrows) *is still present.* A–F: ×82.

flammation of the spinal cord but persists during WD of the crushed optic nerve. KS represents the first marker specific for immunologically resting microglia, which is differentially regulated during immune-mediated and noninflammatory CNS disease. By contrast, other microglial surface molecules such as MHC class II antigens and complement receptors are expressed very weakly by microglia of the nor-

Figure 1. *Downregulation of KS IR during immune-mediated CNS inflammation. Immunocytochemistry on spinal cord sections for KS* (A–D), *macrophages* (E *and* F) *and T cells* (G *and* H). *Numerous KS-positive microglial cells are found in normal controls* (A *and* B) *and myelin-immunized presymptomatic rats on day 11 p.i.* (C) *that are devoid of inflammatory infiltration* (E *and* G). *Symptomatic animals on day 15 display strong infiltration by ED1-positive macrophages* (F) *and T cells* (H) *and KS IR is abolished* (D). A, C–H ×33. B: ×330.

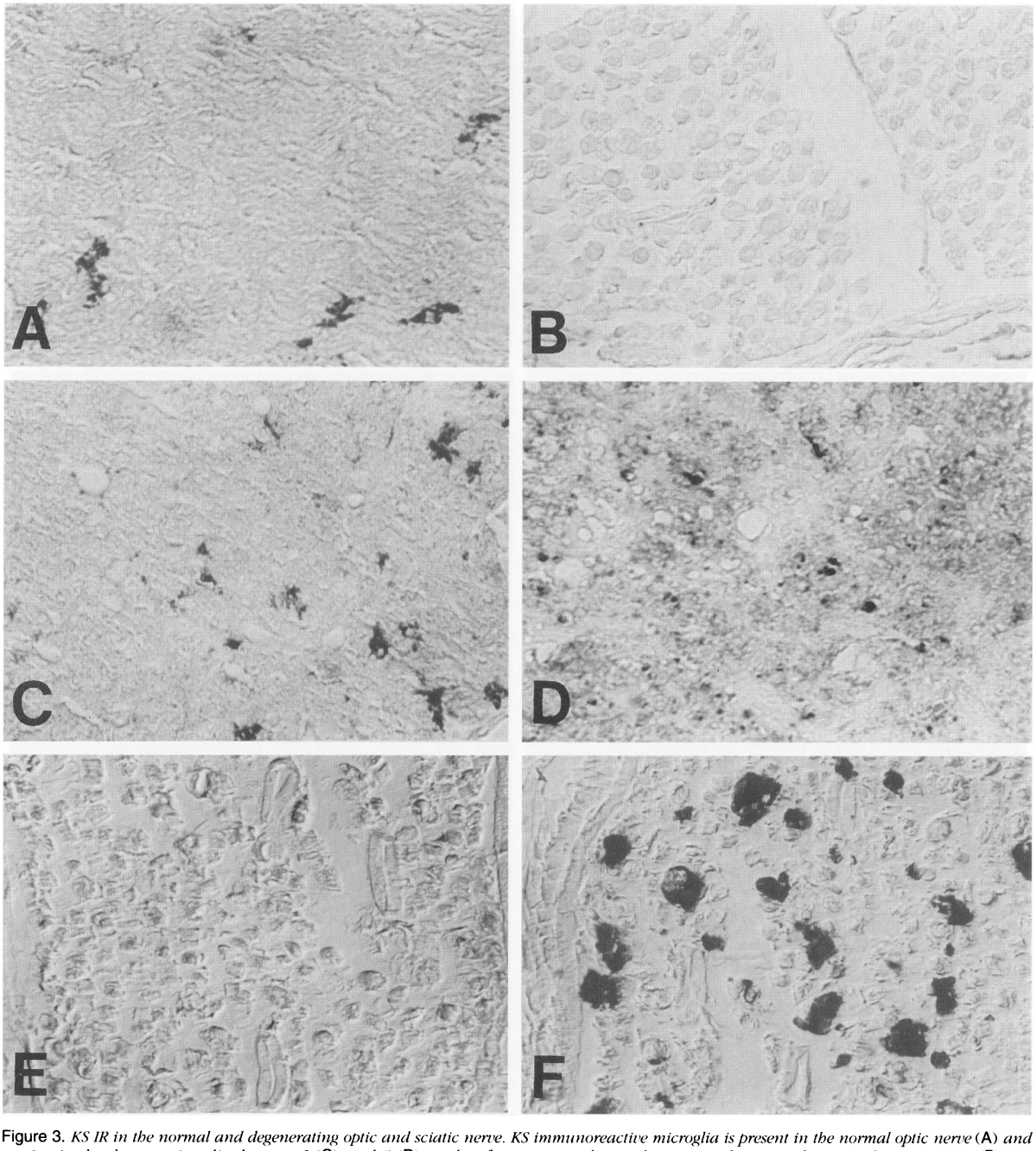

Figure 3. *KS IR in the normal and degenerating optic and sciatic nerve. KS immunoreactive microglia is present in the normal optic nerve* (A) *and persists in the degenerating distal stump 2* (C) *and 8* (D) *weeks after nerve crush. Resident macrophages in the normal sciatic nerve* (B) *are KS-negative. Two weeks after transection the nerve stump is heavily infiltrated by ED1-positive macrophages* (F) *but still no KS IR is detectable* (E). A–F: ×330.

mal CNS[1,5] and increase in EAE and MS[2,6] but also after optic nerve crush[7] and facial nerve axotomy,[5] and in various neurodegenerative processes. Whereas a pronounced macrophage/microglia response is a common characteristic of all these conditions, significant infiltration by T cells is only found in EAE and MS. This suggests that a T cell-derived cytokine could be responsible for the downregulation of KS in EAE. A possible candidate is interferon-γ, which is expressed transiently in EAE,[15] whereas other proinflammatory cytokines such as interleukin-1[11] and tumor necrosis factor-α[16] are equally found in immune-mediated and nonimmune disorders of the nervous system. At present, it remains an open question whether the downregulation of KS expression in EAE is due to decreased biosynthesis, changes in the glycosaminoglycan side chains, or increased degradation of microglial

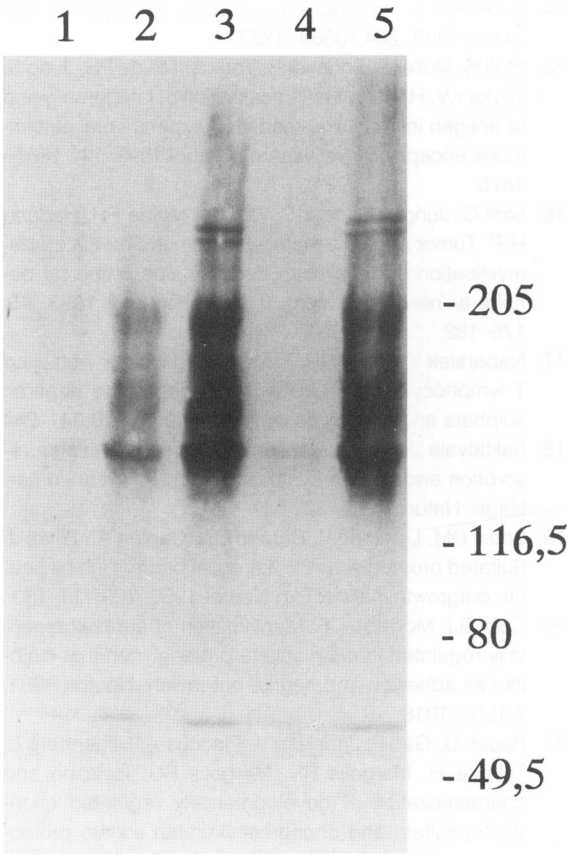

Figure 4. *Immunoblot analysis of 5D4-associated KSPGs. Spinal cord extracts were separated by 4 to 10% SDS-PAGE, transferred to nitrocellulose, and stained using MAb 5D4. Lanes 3 and 5 from normal controls demonstrate heterogenous material mainly located between the 116.5- and 205-kd markers with some additional weak bands in the higher and lower molecular weight range. KS-immunoreactive bands are gradually decreasing in symptomatic EAE animals with mild clinical disease (14 days p.i., lane 2) and severe disease (14 and 15 days p.i., lanes 1 and 4, respectively). Total protein on each lane was 80 μg.*

KSPG. Activated lymphocytes and inflammatory cytokines have the capacity to downregulate biosynthesis as well as to induce degradation of PGs in various forms of tissue inflammation.[17,18]

Our immunocytochemical findings could be further substantiated by immunoblot analysis. In spinal cord extracts from normal rats MAb 5D4 stains a heterogenous set of proteins, which mainly range between 140 and 200 kd. These bands are strikingly reduced in extracts from EAE animals indicating that they represent the relevant microglial protein antigens associated with the 5D4 epitope. The new KSPGs described here differ from other known CNS KSPGs by their apparent molecular weight and spatiotemporal pattern of expression.[19–22]

Another important aspect of the present study relates to the different levels of KS expression in the normal CNS and PNS of adult rats. The 5D4 epitope is present on numerous microglial cells in the spinal cord and a smaller subpopulation in the brain, but is not detectable on resident macrophages of the PNS. Although not definitively proven, both types of resident tissue macrophages are believed to differentiate from a common bone marrow-derived progenitor cell. With respect to their rapid turnover from the bone marrow and putative antigen-presenting cell function *in vivo*, PNS resident macrophages more closely resemble another type of resident CNS macrophage, the perivascular cell.[8,23,24] In contrast to these cell types the parenchymal microglia appears to be an extremely downregulated form of tissue macrophage, possibly because of the special microenvironment in the CNS. Our data suggest that high levels of KSPG expression correlate with a downregulated immunophenotype of resident macrophages/microglia in the nervous system. Interestingly, the macrophage response to axonal damage occurs rapidly in the PNS where no KSPG is detectable but is delayed in the CNS.[9] In view of the rapid macrophage response during EAE coincident with the disappearance of KSPGs, it is conceivable that persistent KSPG expression after optic nerve crush delays an effective macrophage activation during WD.

PGs are critically involved in many regulatory cell-cell and cell-matrix interactions.[14] An intriguing example in the immunological context is the chondroitin sulfate form of the invariant chain, which is expressed associated with MHC class II on the surface of antigen-presenting cells and stimulates T cell responses through interaction with CD44.[25] Based on our present results it is tempting to speculate that microglial KSPGs represent a novel type of immunomolecule that negatively modulates immunological macrophage functions in the CNS.

Acknowledgments

The authors thank Annette Tries and Birgit Blomenkamp for expert technical assistance and Ute Vollmer for photographic work.

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