American Journal of Pathology, Vol. 140, No. 3, March 1992

Immunologic Reactions in Amyotrophic Lateral Sclerosis Brain and Spinal Cord Tissue

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Expression of proteins associated with immune function was investigated immunohistochemically in postmortem brain and spinal cord of patients with amyotrophic lateral sclerosis (ALS). Reactive microglia/macrophages displaying high levels of leukocyte common antigen (LCA), the immunoglobulin receptor FcγR1, lymphocyte function associated molecule-1 (LFA-1), the complement receptors CR3 and CR4, the class II major histocompatibility complex molecules HLA-DR, HLA-DP and HLA-DQ and common determinants of the class I HLA-A,B,C complex were abundant in affected areas in ALS. These areas included the primary motor cortex, motor nuclei of the brain stem, the anterior horn of the spinal cord, and the full extent of the corticospinal tract. A significant number of T lymphocytes of the helper/inducer (CD4+) and cytotoxic/suppressor (CD8+) subtypes were observed marginating along the walls of capillaries and venules and extending into the parenchyma of affected areas. Clusters of complement activated oligodendroglia as well as degenerating neurites positive for C3d and C4d were frequently detected in ALS-affected areas. These data provide evidence of immune-effector changes in ALS. They are consistent with an autoimmune or slow virus theory of the disorder, but may reflect only secondary changes. (Am J Pathol 1992, 140:691–707)

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder of unknown etiology. Numerous theories have been advanced as to its causation, including abiotrophy,[1] slow viruses,[2,3] autoimmunity,[4] chronic intoxication with heavy metals,[5,6] exposure to neurotoxins,[7] abnormal calcium and phosphate metabolism,[8] and alterations in neurotransmitter receptors.[9] In general, theories involving the immune system have not been highly regarded because pathologic investigations using classical techniques have failed to provide convincing evidence of either a humoral or cell mediated immune reaction.[10] However, there are numerous recent reports of anti-neuronal antibodies in the serum or CSF of at least some cases with motor neuron disease.[11–20] In addition, there are a few reports of an inflammatory response in ALS based on the application of newer methods.[21–24] Using sensitive immunohistochemical techniques, we have studied the expression of proteins associated with immune function in postmortem tissue from a series of ALS cases. We show evidence for the appearance of reactive microglia/macrophages expressing MHC glycoproteins and upregulated levels of immunoglobulin and complement receptors in affected ALS tissue. We also show evidence of infiltration of T lymphocytes of both the helper/inducer and cytotoxic/suppressor classes. Finally, we show evidence of complement proteins attached to damaged neurites and oligodendroglia. Particular emphasis was placed on changes in the precentral gyrus and spinal cord as examples of upper and lower motor neuron lesioned areas although extensive pathology was noted through the full extent of the corticospinal tract and in brain stem motor nuclei.

Materials and Methods

Tissue Collection

Thirteen autopsied human brains and spinal cords obtained within 3–15 hours after death were employed in the study: 5 of the brains and the spinal cords came from

Supported by grants from the Amyotrophic Lateral Sclerosis Society of Canada, the American Health Assistance Foundation, the Alzheimer Society of British Columbia, and the Medical Research Council of Canada.

Accepted for publication October 22, 1991.

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cases without neurologic disorder (age range, 67–100; 78.6 ± 5.9) and 8 came from cases of ALS (4 male, 4 female, age range, 47–81; 68.6 ± 3.6) (Table 1). All patients with ALS represented cases of sporadic onset, except one patient whose mother and a maternal aunt both died of ALS in their forties. No other relatives were known to have neuromuscular disease. The total length of disease from the onset of symptoms until death varied from 21 to 96 months. All patients fulfilled clinical and electromyographic criteria for ALS premortem with the diagnosis being confirmed postmortem by observation of losses of both lower and upper motor neurons, as well as compromise of the corticospinal tract.

Immunohistochemistry

Small blocks of brain and spinal cord tissue were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 days at 4°C and transferred to a cold solution of 15% sucrose in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Sections were cut on a freezing microtome at 30 μm thickness, collected in buffered 15% sucrose solution, and stored at 4°C until used. Some unfixed blocks were immediately frozen on dry ice and sectioned in a cryostat. Unfixed cryostat sections were mounted on glass slides, air dried, and fixed in acetone for 10 minutes. Sections were first pretreated with 0.3% H_2O_2 for 30 minutes to eliminate endogenous peroxidase, and then placed in a blocking solution of 10% skim milk powder at 4°C overnight. After these pretreatments, sections were incubated with primary antibody for 72 hours in the cold. The primary antibody selected (Table 2) was diluted in PBS containing 0.3% Triton-X100, and, as a blocking solution for the secondary antibody, 10% normal serum of the species in which the secondary antibody was raised. After incubation with primary antibody, sections were next treated with biotinylated secondary antibody (Vector Lab) for 2 hours at room temperature, followed by incubation in the avidin-biotinylated HRP complex (Vector Lab) for 1 hour. Peroxidase labeling was detected in most instances by incubation with a solution containing 0.01% 3,3′-diaminobenzidine (DAB), 0.6% nickel ammonium sulfate, 0.05M imidazole and 0.00015% H_2O_2 to give a purple precipitate. With weakly staining sections, especially HLA-A,B,C, the DAB concentration was increased to 0.02% and H_2O_2 to 0.003%. The intensity of staining was monitored by removing the sections from the DAB solution periodically, mounting them on a slide, and inspecting them under a microscope. An incubation time of 5–15 minutes was usually sufficient. The reaction was terminated by transferring the sections to a solution of PBS. Sections were mounted on glass slides, dehydrated, and coverslipped with Entellan (Merck). For double immunostaining, sections were put through a second, slightly modified cycle. After completion of the first cycle, sections were treated for 30 minutes with 0.5% H_2O_2 solution in PBS to destroy residual peroxidase activity. The second immunohistochemical cycle was then carried out in a similar manner to the first, except that nickel ammonium sulfate was omitted from the DAB solution, yielding a brown precipitate in the second cycle. Some sections were subsequently stained by the Bielschowsky silver method. Others were counterstained with neutral red or cresyl violet. Sections used for cell counting were stained only with cresyl violet.

Three types of controls were used: elimination of the primary antibody; substitution of rabbit serum hyperimmunized with tobacco mosaic virus; and use of a mouse monoclonal antibody indifferent to brain antigens. No positive staining of either normal or ALS brain was obtained in control sections.

Table 1. *Cases Used in This Study*

Case	Sex	Age	Diagnosis	Duration (mo)	Symptom*	Motor neuron density		Postmortem (hr)
						Cortex†	Ant. horn‡	
1	M	67	Cancer			180	132	7
2	F	100	Cancer			169	196	4
3	M	73	AMI			189	216	10
4	M	82	AS			187	222	12
5	M	71	AMI			191	250	3
6	F	75	ALS (s)	30	B+E+R	113	103	9
7	M	67	ALS (s)	34	B+E+R	66	56	10
8	F	47	ALS (f)	48	E+B+R	70	36	3
9	M	71	ALS (s)	29	E+B+R	53	57	6
10	F	65	ALS (s)	21	B+E+R	44	24	7
11	M	75	ALS (s), AMI	21	E+B+R	78	28	3
12	M	68	ALS (s)	96	E+B+R	52	41	12
13	F	81	ALS (s)	30	B+E+R	140	61	15

* The course of clinical symptoms of muscle weakness is shown. B, bulbar symptoms; E, weakness of extremities; R, respiratory muscle weakness.

† Layer V pyramidal neurons in 50 fields, each 100 μm × 30 μm thickness.

‡ Anterior horn cells in six hemisections of the cervical cord.

AMI, acute myocardial infarction; AS, arteriosclerosis; ALS (s) and ALS (f), sporadic and familial case of ALS, respectively.

Table 2. *Antibodies Used with Their Types and Dilutions*

Antigen	Antibody (designation)	Source	Type	Dilution
LCA (CD45)	2B11 + PD7/26	Dakopatts	monoclonal*	1:100
FcγR1 (CD64)	32.2	Medarex	monoclonal†	1:1000
LFA-1 (CD11a)	MHM24	Dakopatts	monoclonal*	1:100
CR3 (CD11b, Mac-1)	Bear-1	Sanbio	monoclonal*	1:100
CR4 (CD11c, p150.95)	Leu-M5	Becton-Dickinson	monoclonal†	1:10
HLA-DR	HB104	ATCC	monoclonal*	1:1000
HLA-DP	Leu-10	Becton-Dickinson	monoclonal†	1:500
HLA-DQ		Becton-Dickinson	monoclonal†	1:500
HLA-A,B,C	HB116	ATCC	monoclonal*	1:500
	+HB120	ATCC	monoclonal*	1:100
ICAM-1	PR1/1.1.1	Boehringer Ingelheim‡	monoclonal†	1:5000
C3d		Dakopatts	rabbit polyclonal	1:20000
C4d		Quidel	monoclonal†	1:1000
Fraction Bb		Cytotech	monoclonal†	1:100
Properdin		Cytotech	monoclonal†	1:100
CD4	NUTH/1	Nichirei	monoclonal*	1:100
CD4	T4‖	Dakopatts	monoclonal*	1:50
CD8	T8	Dakopatts	monoclonal*	1:1000
Collagen IV		Southern Biotech	goat polyclonal	1:20000
GFAP		Dakopatts	rabbit polyclonal	1:10000

* Mouse monoclonal antibody (supernate).
† Mouse monoclonal antibody (purified IgG from ascites).
‡ Courtesy Dr. R. Rothlein.
‖ Worked only in fresh-frozen, acetone fixed tissue.

Primary Antibodies and Their Antigens

The primary antibodies used in this study and their antigens are listed in Table 2. The antibodies are all commercially available. The source, type of antibody, and dilution employed are given in the table. The antigens are mostly functionally important leukocyte and complement proteins. Leukocyte common antigen (CD45), as the name implies, is found on all leukocytes and is believed to modify signal transduction via tyrosine phosphatase activity.[25] FcγR1 (CD64) is an immunoglobulin receptor, which is highly localized to monocytes. It occurs in low concentration on granulocytes, but not on other leukocyte cell lines.[26] Its function is to bind cell surfaces to antigen-antibody complexes. The β-2 integrins (CD11a, CD11b and CD11c), also known as leukocyte adhesion molecules,[27] bind leukocytes to cell surface and matrix proteins. CD11a (LFA-1) is found on activated lymphocytes, NK cells, monocytes, and granulocytes.[25,28] CD11b (CR3, Mac-1)[29] and CD11c (CR4, P150.95)[30] are mainly expressed on phagocytic cells and NK cells. The function of LFA-1 is to bind appropriate leukocytes to ICAM-1, whereas the function of CR3 and CR4 is to bind appropriate leukocytes to complement proteins. The group II major histocompatibility complex glycoproteins HLA-DR, HLA-DP, and HLA-DQ are expressed on immunocompetent cells, whereas the group I MHC complex HLA-A,B,C is expressed by many cell types. They serve, respectively, as recognition molecules for T-helper/inducer (CD4 positive) and T-cytotoxic/suppressor (CD8 positive) lymphocytes.[25] C4d is a chemically bound fraction of C4 which is found only when the classical complement pathway is activated. Properdin and fraction Bb of Factor B are associated only with the alternative complement pathway. C3d is a chemically bound fraction of C3 which is found when either the classical or alternative pathway is activated.[31] Collagen type IV is a capillary basement membrane protein,[32] whereas GFAP is found exclusively in astrocytes.[33]

Results

Neuronal Counts

Neuronal counts in layer V of the motor cortex and in the anterior horn of the spinal cord were carried out for each control and each ALS case. Pyramidal neurons of layer V were counted in fifty fields of 100 μm^2 area (5000 μm^2, 30 μm thickness) and anterior horn neurons in 6 hemisections of the cervical cord (30 μm thickness) were counted for each control and each ALS case. In the motor cortex, pyramidal cell counts in the normals ranged from 169 to 191 (mean, 183.2 ± 8.9) whereas those in the ALS cases ranged from 44 to 140 (mean, 77.0 ± 33.2). There was no overlap, and the difference was highly sig-

nificant ($P < .0001$). In the spinal cord, anterior horn cell counts in the normals ranged from 132 to 250 (mean, 203.2 ± 44) while those in the ALS cases ranged from 24 to 103 (mean, 50.7 ± 25.2). Again there was no overlap and the difference was highly significant ($P < .0001$)

Microglial Surface Proteins

In normal tissue, microglia constitutively expressed LCA.[33–35] The appearance of resting microglia in control cases is shown for the gray matter (Figure 1A), and white matter (Figure 1B) of the precentral gyrus; and the lateral funiculus (Figure 1C) and anterior horn (Figure 1D) of the spinal cord. These same areas are shown in Figure 1E–1H for a typical ALS case. Both upper (Figure 1E) and lower (Figure 1H) neuron areas were dominated by strongly stained reactive microglia, indicating morphologic change and upregulation of LCA. The same was true for the subcortical white matter (Figure 1F) and lateral funiculus (Figure 1G) except that the cells were lipid laden, fitting the description of foamy macrophages. In addition, significant numbers of even more intensely stained leukocytes were observed (arrows), sometimes gathered in and around capillaries.

Microglia also constitutively express the immunoglobulin receptor FcγR1 as well as β-2 integrins, which are receptors for LFA-1 and certain complement proteins.[25–35] Examples are shown in Figure 2. Figure 2A illustrates staining of control precentral gyrus subcortical white matter and Figure 2B control lateral funiculus for FcγRI. Figure 2C shows staining of control precentral gyrus gray matter for the β-2 integrin CD11b (CR3). Comparable staining of these same areas is shown for an ALS case in Figure 2E–G. The staining in the ALS case is dominated by reactive microglia/macrophages as in Figure 1. The staining is also more intense, indicating upregulation of the receptors. There was, however, one significant difference between staining for LCA and FcγRI. In the case of FcγRI staining, almost no leukocytes were observed, indicating that the leukocytes were not of monocytic origin.

Figure 3 shows staining of the gray matter of the precentral gyrus of a normal and an ALS case for HLA-DR (Figure 3A, B), HLA-DP (Figure 3C, D), HLA-DQ (Figure 3E, F) and HLA-A,B,C (Figure 3G, H) as well as the anterior funiculus of the spinal cord for HLA-A, B, C (Figure 3I, J). For each of the group II MHC glycoproteins, few positive microglia were stained in normal tissue, whereas in ALS tissue many reactive microglia were intensely stained. For the group I HLA-A, B, C complex, only capillaries were stained in normal tissue, whereas in ALS tissue reactive microglia were also stained, although weakly in comparison with the intense capillary staining.

Figure 4 compares HLA-DR staining of normal and ALS spinal cord. Figure 4A and B shows photomontages of hemisections of the spinal cord of a normal case and an ALS case. The normal case shows few HLA-DR positive microglia, whereas the ALS case shows many intensely stained reactive microglia throughout the cord, especially in the regions of the lateral and anterior funiculi, as well as in the anterior horn. Figure 4C and D shows the lateral funiculi under higher power. Again the normal case shows few HLA-DR positive microglia whereas the ALS case shows many lipid-laden, HLA-DR positive cells. Such differences became even more obvious when sections were counterstained with oil red O. The lipid-containing microglia/macrophages developed an intense cherry red color that enhanced their staining. This is illustrated by comparing Figure 5A with Figure 5B.

Complement Proteins and ICAM-1

Clusters of C3d- and C4d-coated fibers were seen in the affected areas in ALS (Figure 6) and were frequently associated with oligodendroglia. We have previously described these as complement-activated oligodendroglia (CAO).[36] A large number of CAO were observed in the gray matter of the precentral gyrus, especially in the layers III and V-VI of the primary motor cortex (Figure 6A); some, but fewer, CAO were seen in the white matter of the precentral gyrus (Figure 6B) and in the anterior horn (Figure 6C) of the spinal cord. Dotty deposits of C4d in the lateral funiculi appeared to represent attachments of C4d to the surface of corticospinal fibers running longitudinally (Figure 6D) since spinal sections were cut transversely from the blocks in this study. In control tissues, CAO were rare (Figure 6E, F). A few were found in the gray matter of the precentral gyrus of some cases, but there were none in the white matter of the precentral gyrus or in the spinal cord (data not shown). Antibodies to fraction Bb of factor B (Figure 6G) and factor P (Figure 6H) did not stain ALS tissue. CR4-positive microglia were often seen surrounding the C4d-positive CAO in the affected areas of ALS cases as well as near degenerating neurons and their axons.

This relationship was confirmed by examination of sections stained for C4d and counterstained by the Bielschowsky silver method. Degenerating axons were revealed by Bielschowsky staining, many of which were also positive for C4d.

Staining for the intercellular adhesion molecule-1 (ICAM-1), the ligand for LFA-1, was found in capillaries and venules in normal tissue (Figure 7A), and in ALS cases, as diffuse deposits around vessels. Figure 7B demonstrates this in layers V-VI of the primary motor cortex and Figure 7C in the anterior horn of the spinal cord. The precentral subcortical white matter and the pyrami-

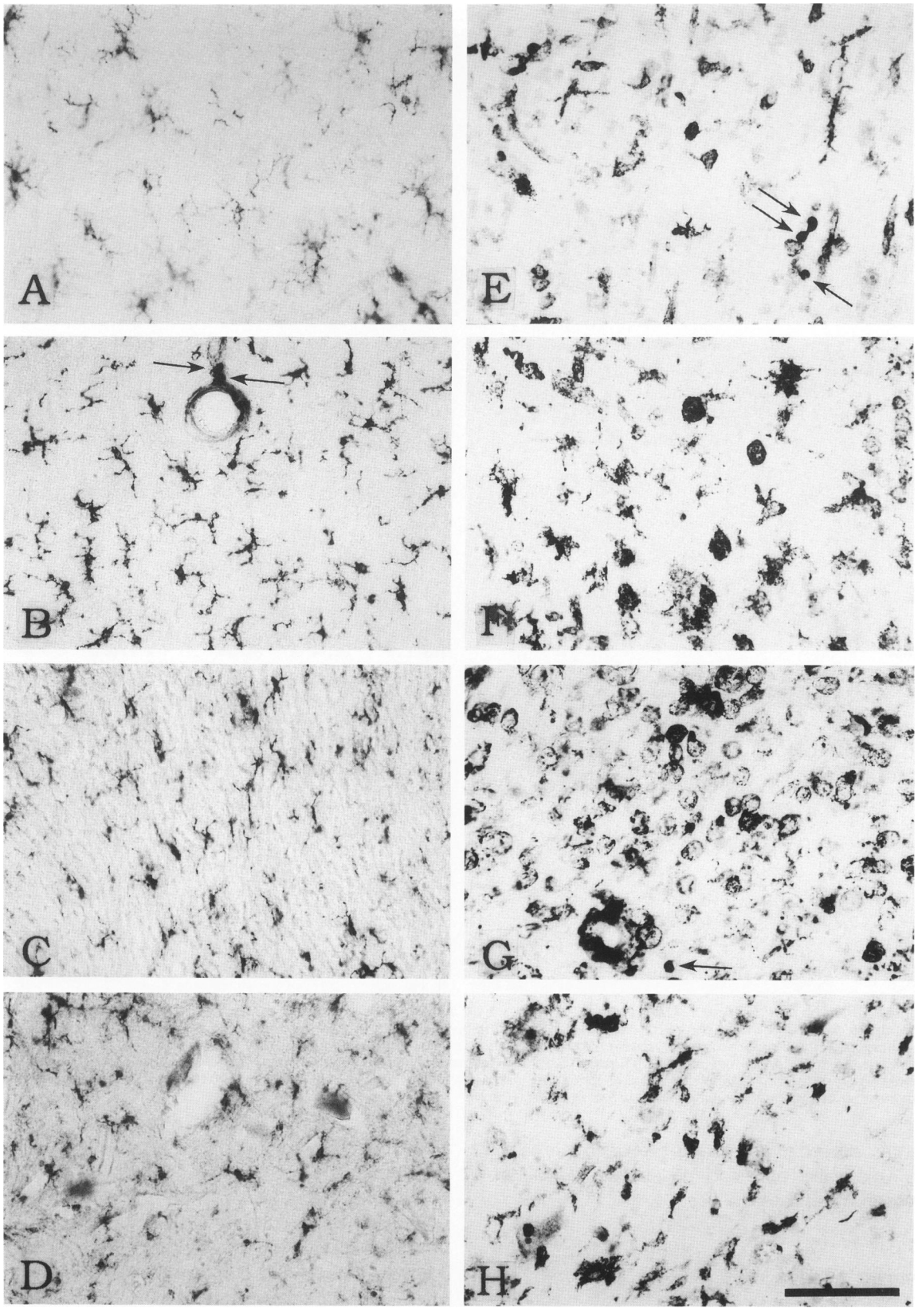

Figure 1. *Immunostaining for leukocyte common antigen (LCA) in control* (A–D) *and ALS* (E–H) *tissue. Resting microglia are observed in the control precentral gray matter* (A), *the subjacent white matter* (B) *the spinal cord lateral funiculus* (C), *and the anterior horn* (D). *A few leukocytes are also stained (arrows), including some in a venule in* (B). *In ALS tissue, many reactive microglia are observed in the precentral gray matter* (E). *In the subjacent white matter, these reactive microglia become lipid-laden* (F). *Similar lipid-laden cells are observed in the lateral funiculus* (G). *In the anterior horn* (H), *the reactive forms are more similar to those in the cortical gray matter. Again, arrows point to leukocytes. All photomicrographs are at the same magnification. Bar in* (H) = *100 μm.*

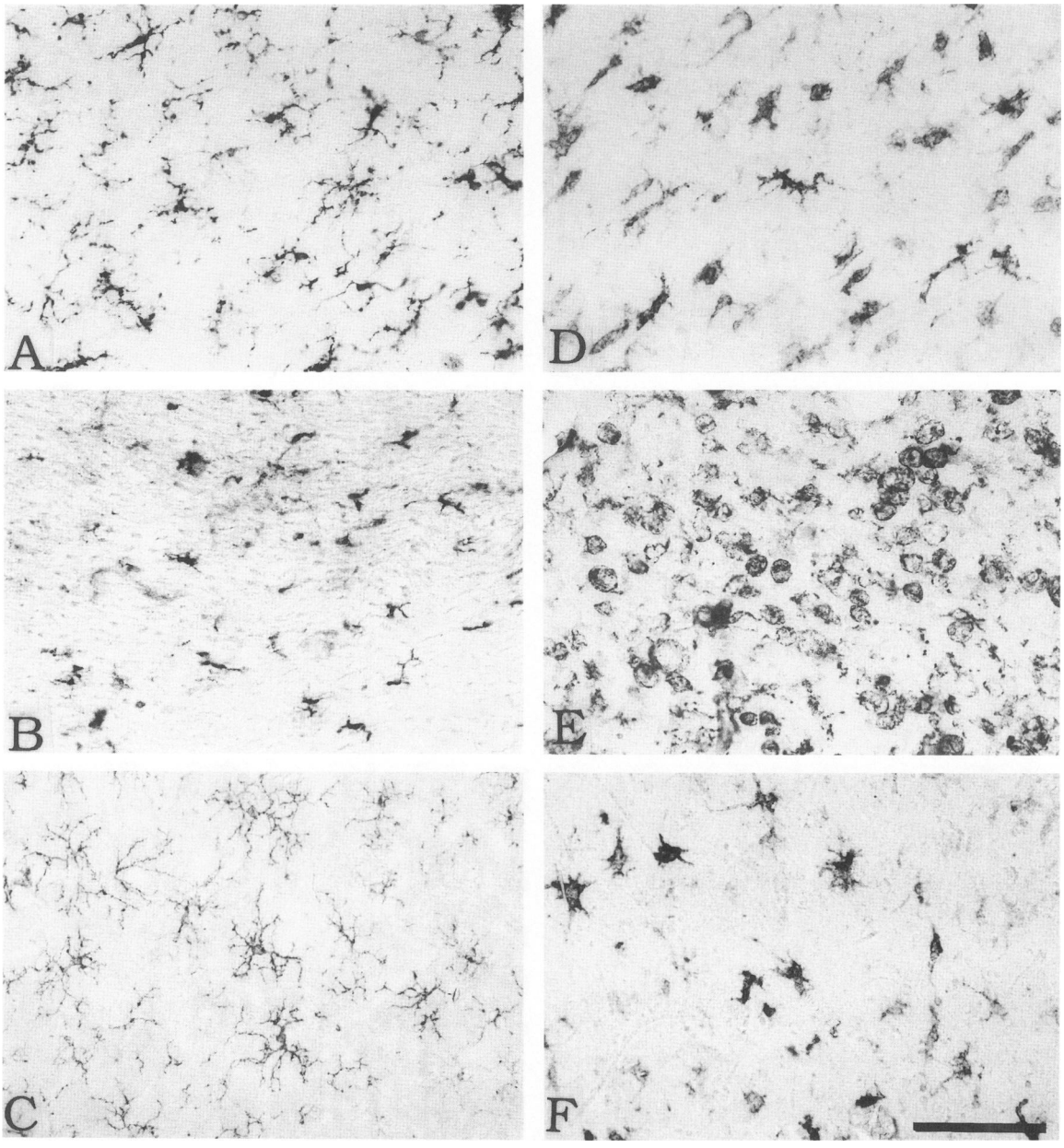

Figure 2. *Immunostaining of control* **(A–C)** *and ALS* **(D–F)** *tissue for the phagocytic receptor FcγR1 and the β-2 integrin CR3 (CD11b). The staining is highly similar to that observed for LCA;* **(A, D)** *precentral gyrus gray matter of a control and an ALS case; and* **(B, E)** *the lateral funiculus of the spinal cord of a control and an ALS case stained for FcγRI. Notice the many lipid-laden cells in the lateral funiculus* **(E)** *and the absence of FcγRI positive leukocytes in ALS tissue;* **(C, F)** *immunostaining of the precentral gray matter of a control and ALS case for CR3 (CD11b). Again, control tissue shows staining for resting microglia* **(C)**, *while many reactive microglia appear in ALS tissue* **(F)**. *All photomicrographs at the same magnification. Bar in* **(F)** = *100 μm.*

dal tract showed similar staining. In addition, rare cells in the white matter, apparently glial, showed ICAM-1 expression in ALS cases.

Leukocyte Staining

A significant number of lymphocytes positive for CD4 (Figure 8A, B) and CD8 (Figure 8C, D) were observed marginating along the walls of capillaries and venules and invading the parenchyma of inflamed areas in sections of ALS tissue. Some of the sections were immunostained for collagen type IV in a second cycle to reveal the location of capillaries[32] after first-cycle immunostaining for CD4 or CD8 (e.g., Figure 8A–F). The majority of the T lymphocytes belonged to the CD8-positive suppressor/cytotoxic T-cell subset, but CD4-positive helper/inducer T cells were also found. No T lymphocytes were

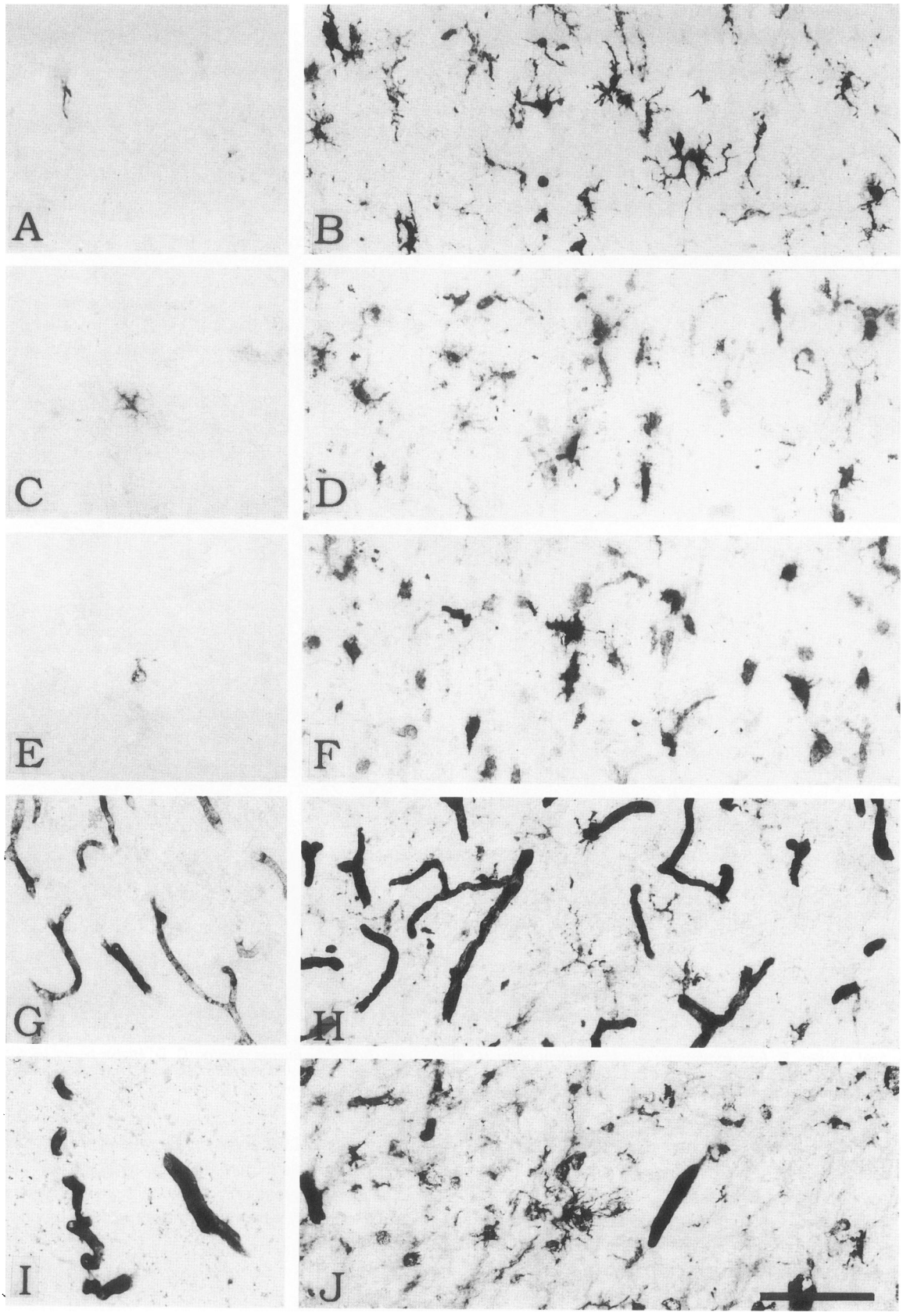

Figure 3. *Immunostaining of control and ALS tissue for MHC class II* (A–F) *and MHC class I* (G–J) *antigens;* (A) *and* (B) *compare staining of control* (A) *with ALS* (B) *precentral gray matter for HLA-DR. Only one cell can be seen in* (A) *while many reactive microglia are visible in* (B); (C) *and* (D) *show a similar comparison of staining for HLA-DP, and* (E) *and* (F) *for HLA-DQ. A somewhat different pattern of staining is seen for HLA* (A, B, C). *In both control* (G) *and ALS* (H) *precentral gray, capillaries are intensely stained. In ALS precentral gray, reactive microglia are also lightly stained. Similarly, in the anterior funiculus of the spinal cord* (I, J), *staining for HLA* (A, B, C) *in control tissue* (I) *shows only staining of capillaries, while in ALS tissue* (J), *many reactive microglia are also visible. All photomicrographs are at the same magnification. Bar in* (J) = *100 μm.*

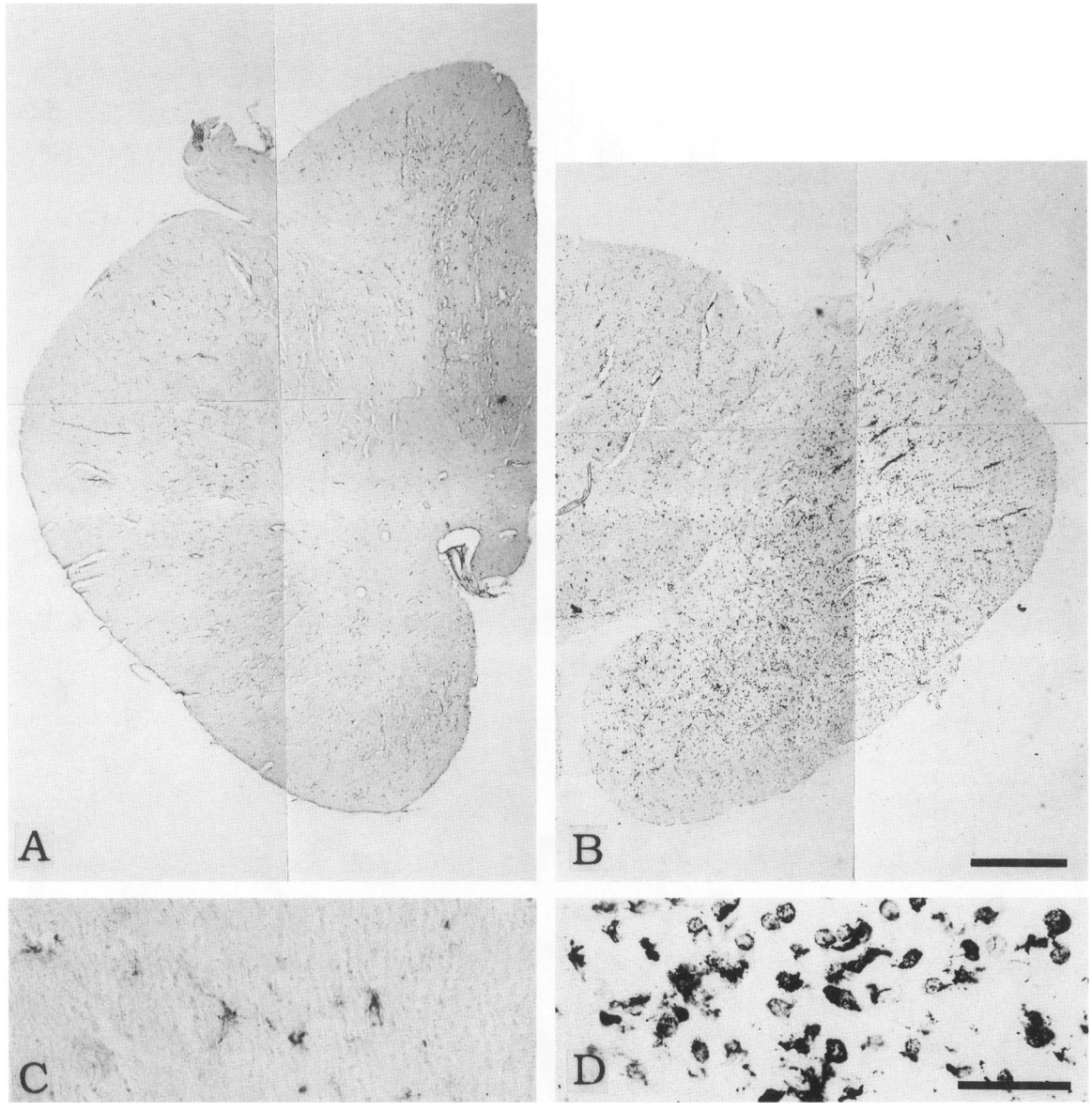

Figure 4. *Immunostaining of the cervical cord for HLA-DR.* **A**: *Low-power photomontage of a hemisection of a control cervical cord. Notice the light staining.* **B**: *Immunostaining of a hemisection of an ALS spincal cord. Notice the intense staining.* **C, D**: *Higher power photomicrographs showing the control* (C) *and ALS* (D) *lateral funiculus. Again, intense staining is observed in the ALS case;* (A, B) *same magnification; bar in B = 1 mm;* (C, D) *same magnification; bar in* (D) *= 100 μm.*

seen in the matrix of control brains (Figure 8E, F) or unaffected areas of ALS brain, and only occasional T lymphocytes were seen in vessels.

For positive control staining of leukocytes, lymph node and spleen from a myocardial infarct case were processed in an identical manner to brain tissue and stained for LCA, CD4, CD8, and FcγRI. Both tissues showed large numbers of LCA, CD4, and CD8 positive round cells but only limited numbers of FcγRI positive round cells. The numbers of CD4 positive cells (Figure 8G) were comparable to the numbers of CD8 positive cells (Figure 8H), which is different from the ratio noted for ALS tissue. The numbers of LCA positive cells were even larger than CD8 positive cells (Figure 8I) since all leukocytes carry this antigen, whereas only a few cells were positive for FcγRI (Figure 8J), which is consistent with low monocyte levels in blood.

Leukocyte Counts

To estimate the relative concentration of leukocyte populations in ALS and control tissue, serial sections of the precentral gyrus and cervical cord were stained for LCA,

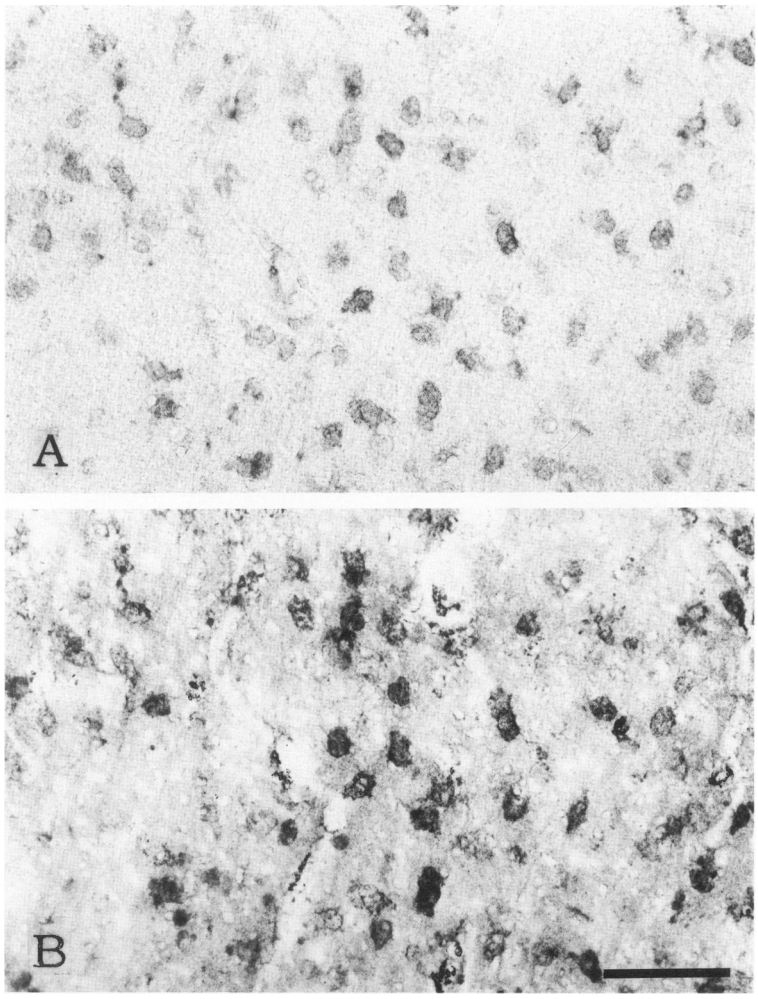

Figure 5. *Immunostaining of the precentral gyrus white matter for HLA-DR* (A) *and HLA-DR counterstained with oil red O* (B) *in ALS tissue. Many reactive microglia are visible in* (A), *with the staining of most becoming much intensified in* (B). *In the original slides, the HLA-DR staining was purple. The oil red O produced an intense cherry red color within most cells;* (A) *and* (B) *are at the same magnification. Bar in* (B) = *100 μm.*

CD8, CD11a (LFA-1), CD11b, CD11c, and FcγRI. The number of positively staining round cells was counted in cortical gray matter areas of 32–41.8 mm^2 and in hemisections of the thoracic spinal cord of areas 22.9 to 30.3 mm^2 for each antigen.

Table 3 shows the the results as numbers of cells counted per area, and as numbers per mm^3 of tissue. In the control spinal cord, the total leukocyte count by LCA staining was 167 cells per mm^3. Only a few cells stained positively for the various subtypes that were tested. In ALS spinal cord the counts were increased several fold. The total LCA count was 872/mm^3, the CD8 count was 917/mm^3 and the CD11a count, 863/mm^3. In the precentral gray of the normal case the counts were comparable to those in normal spinal cord. In the ALS precentral gyrus, the counts were again elevated, but not to the same extent as in the spinal cord. The LCA count was 175, the CD8 count was 108 and the CD11a count was 161. The FcγRI count was low in both normal and ALS tissues, varying from 6–10/mm^3.

GFAP

Many intensely staining reactive astrocytes were observed with GFAP staining of ALS precentral gyrus and spinal cord, but not those of control. These GFAP-positive astrocytes were especially evident in layers II and III but also in layers IV and V of the precentral gyrus (Figure 9A), as well as in the subjacent white matter (Figure 9B). No cells with the morphology of GFAP-positive reactive astrocytes expressed HLA-DR, HLA-DP, HLA-DQ, HLA-A,B,C or other microglial markers, nor were any decorated with any complement protein. There were, however, rare class I positive glial cells or glial agglomerates which could not be further identified on the basis of their morphology.

Discussion

ALS is generally described as a disease in which there is selective degeneration of upper and lower motoneurons,

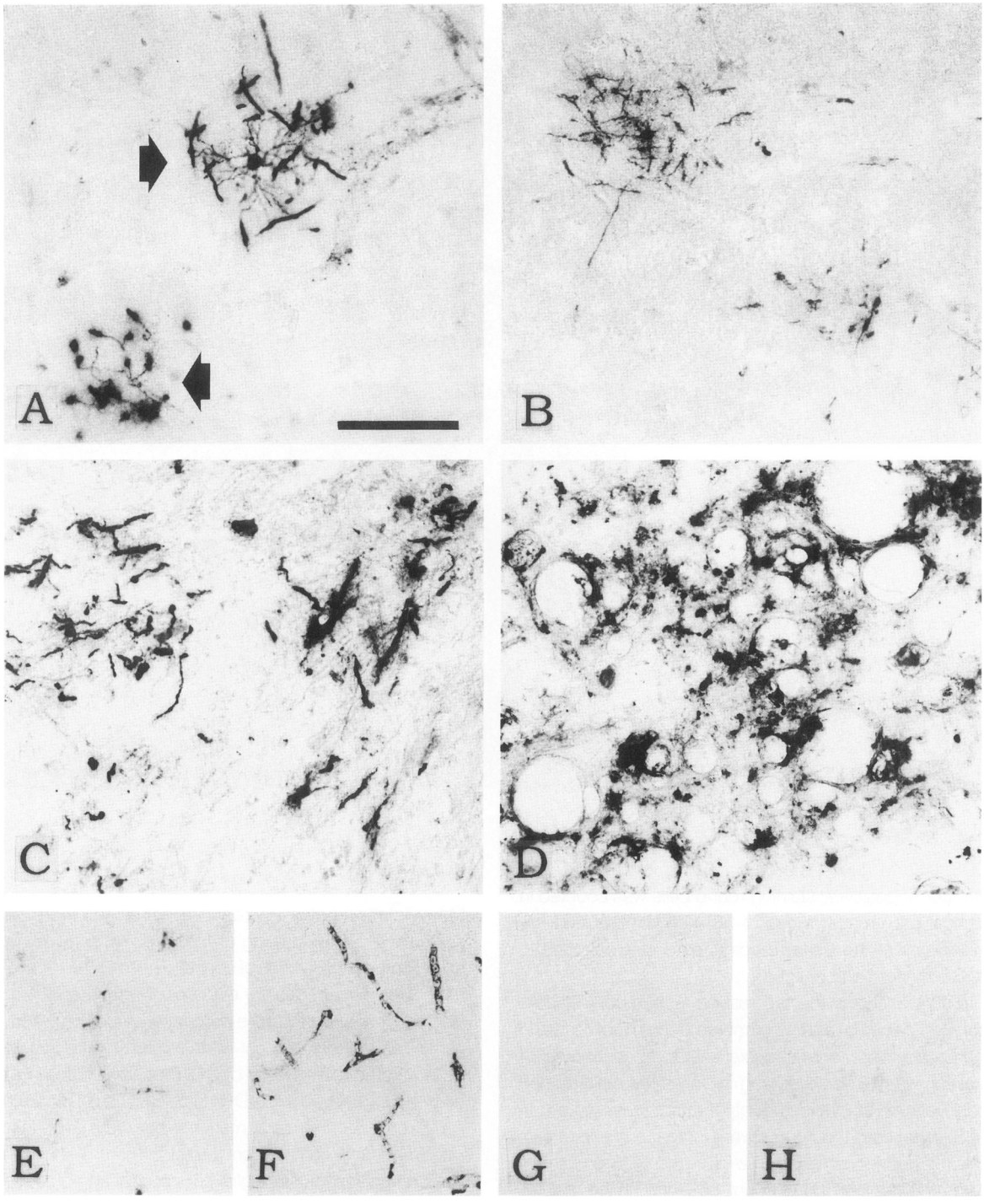

Figure 6. *Immunostaining for complement proteins in ALS tissue.* **A**: *Precentral gyrus gray matter stained for C4d. Notice the complement activated oligodendroglia (arrows).* **B**: *Precentral gyrus immunostained for C3d. Comparable staining to that for C4d is obtained, although staining of residual serum in capillaries is more intense.* **C**: *Immunostaining of the anterior horn of the spinal cord for C4d. Positively staining profiles include apparent neuronal processes.* **D**: *Lateral funiculus of ALS spinal cord immunostained for C4d. Intense staining is obtained. Many structures, possibly related to immunostained long tracts that have been cut in cross-section, are positive.* **E, F**: *Immunostaining of control precentral gyrus gray matter tissue for C4d* (**E**) *and C3d* (**F**). *Only staining in residual serum is observed.* **G, H**: *Immunostaining of ALS precentral gyrus gray matter tissue for fraction Bb of factor B* (**G**) *and properdin* (**H**); *no positive staining is obtained. All photomicrographs at the same magnification. Bar in* (**A**) = 100 μm.

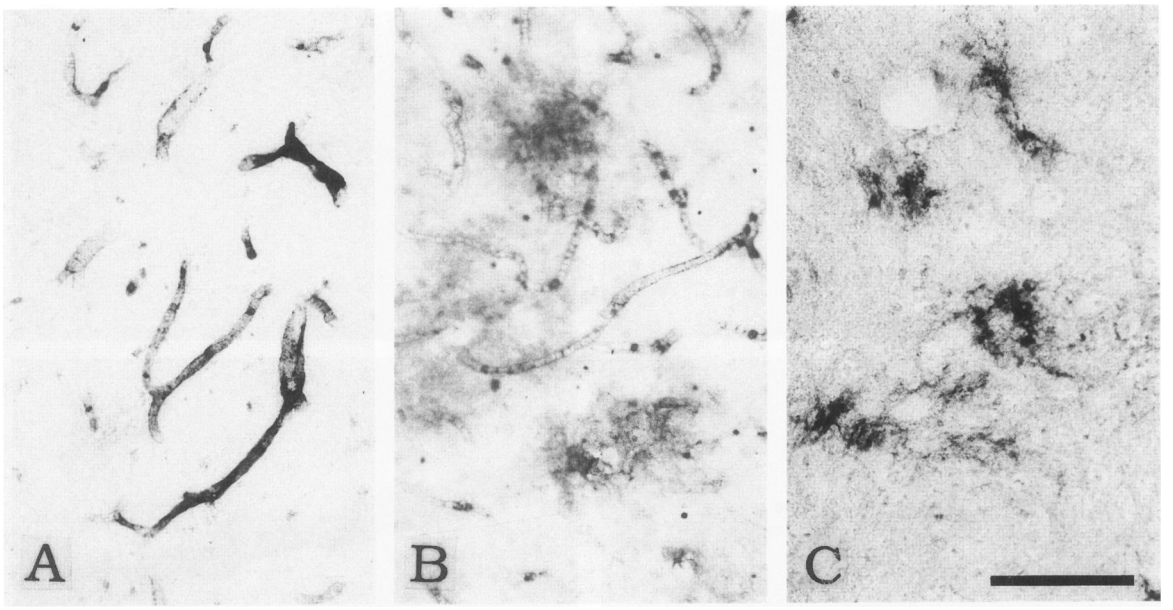

Figure 7. *Immunostaining of control and ALS tissue for ICAM-1.* **A:** *Control precentral gyrus gray matter. Capillaries are intensely stained.* **B:** *Comparable area of an ALS case. In addition to capillaries, diffuse immunostained patches appear in the matrix.* **C:** *Anterior horn of the spinal cord in ALS. Again, diffuse ICAM-1 positive patches are observed. All photomicrographs are at the same magnification. Bar in* (**C**) = *100 μm.*

with only a mild astroglial proliferation occurring as the inevitable accompaniment of neuronal disintegration, and with no evidence of inflammation.[10] This view is based on classical histologic techniques in which tissues are first fixed in formalin and then embedded in paraffin. Such techniques destroy many surface antigens, making important functional proteins undetectable by immunohistochemistry, and obscuring the identification of many significant cells.

In a few recent papers, where more sensitive methods have been employed, changes suggestive of immune effector phenomena have been reported. Troost et al[24] and Lampson et al[22] found T-lymphocyte infiltration and increased expression of MHC class I and class II antigens on dendritic cells and foamy macrophages in ALS spinal cord. We previously reported on a single case of ALS in which striking changes were observed in microglial expression of HLA-DR in the motor cortex and spinal tract.[23] Immunohistochemical staining for this antigen-presenting protein was so intense that areas of pathology in the motor cortex and the corticospinal tract could be identified in tissue sections with the naked eye.

We have now extended this finding to a series of cases, and confirm the findings of Troost et al[24] and Lampson et al[22] regarding T-lymphocytic infiltrates. We detected significant numbers of T4 and T8 cells marginated along capillary walls and free in the tissue matrix in every ALS case. The T8 cells were considerably more numerous than the T4 cells, and were conspicuously clumped together in some venules (Figure 8D).

Our findings agree with those of Lampson et al[22] with respect to the cell types expressing MHC class I and class II glycoproteins, and their distribution in control and ALS spinal cord (Figures 3, 4). Only capillary endothelial cells were positive for class I in control tissue (Figure 3G, I), whereas in ALS tissue some reactive microglia were weakly to moderately stained in the precentral gyrus (Figure 3H). Only rare microglia were stained for MHC class II glycoproteins in control tissue, whereas many reactive microglia/macrophages were positive in ALS tissue.

We also detected upregulated levels of phagocytic receptors and other leukocyte surface proteins on microglia/macrophages. We further identified complement proteins, which are putative ligands for some phagocytic receptors, decorating degenerating neuronal processes and some oligodendroglia.

In normal cortex, brain stem, and spinal cord, we found, as previously reported, that microglia, whether resting or reactive, express constitutively LCA[33] (Figure 1), the FcγRI receptor[34] (Figure 2), and β-2 integrins[35] (Figure 3). These results on constitutive expression of leukocyte markers on microglia provide further information on microglial phenotypic characteristics, linking them with peripheral monocytes.

In ALS-affected tissue, all of these leukocyte surface proteins were sharply upregulated on reactive microglia. The reactive microglia displayed the variety of morphologic forms initially described by Rio Hortega[37] and Penfield,[38] with enlarged cytoplasm and processes that were shortened and thickened. In myelin-rich areas, most of

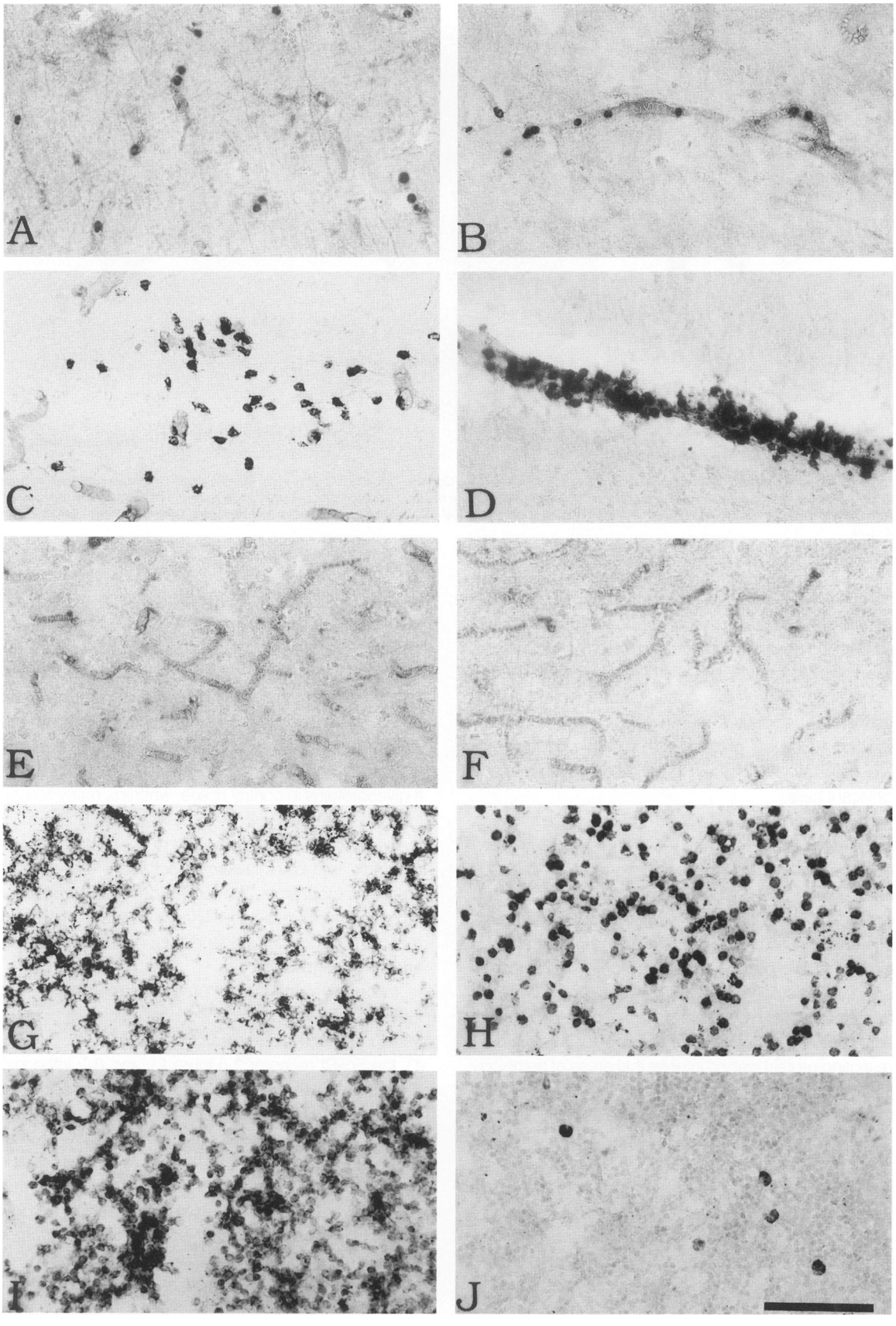
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Table 3. *Round Cell Counts in Spinal Cord and Motor Cortex following Staining for Various Leukocyte Antigens**

	Spinal Cord				Motor Cortical Gray			
	ALS		Control		ALS		Control	
Antigen	Cts/22.9 mm^2	Cts/mm^3	Cts/30.3 mm^2	Cts/mm^3	Cts/32 mm^2	Cts/mm^3	Cts/41.8 mm^2	Cts/mm^3
LCA	599	(872)	152	(167)	168	(175)	123	(98)
CD4	186	(271)	9	(10)	51	(53)	15	(12)
CD8	630	(917)	10	(11)	106	(108)	13	(10)
CD11a	593	(863)	6	(7)	158	(161)	35	(28)
CD11b	98	(131)	29	(31)	91	(95)	56	(45)
CD11c	25	(36)	7	(8)	11	(11)	6	(5)
FcγR1	4	(6)	6	(7)	6	(6)	13	(10)

* Counts were performed on 30 μm serial sections of the precentral gray and thoracic cord of one control and one ALS case. The area counted is shown as mm^2.
Values in brackets are adjusted to counts/mm^3.

the reactive cells were bloated and round, with no processes. Penfield described these cells as the fat granule stage of reactive microglia, but they are usually referred to as foamy macrophages. Some information on the nature of material inside these cells can be inferred from its reaction with oil red O. Enzymatically degraded myelin reacts with oil red O to produce a cherry red color instead of the pale pink of normal myelin.[39] When sections were counterstained with oil red O, cherry red dots appeared on the inside, but not the outside, of these reactive microglia/foamy macrophages. This result is similar to that observed at the margins of multiple sclerotic plaques where a cherry red color appeared inside reactive microglia/macrophages.[39] A continuous gradation from such foamy macrophages to resting microglia in adjacent white matter was observed, suggesting that the morphology of the cell was changing as it phagocytosed greater quantities of myelin. A comparable gradation was observed in ALS, although there was no central plaque.

Although there was no difference in microglial staining using various leukocyte markers, there were substantial differences in round cell staining (Table 3). Only a few such cells were detected in sections from ALS or control tissue stained for FcΓRI. This suggests an absence of monocyte infiltration in ALS. On the other hand, substantial numbers of round cells were detected in sections of ALS spinal cord and precentral gyrus stained for LCA, LFA-1 and CD8. This indicates that an infiltration of activated lymphocytes does take place. The tendency for groups of round cells to accumulate in some venules, but not others, was consistently observed (Fig. 8D). The meaning of this is unclear. The numbers of round cells detected by staining for LCA and CD8 were comparable, which would be expected if the infiltrate is dominated T-cytotoxic/suppressor cells. Smaller numbers of round cells were positive for CD11b and CD11c suggesting the possibility of other lymphocyte populations being part of the infiltrate. Very few FcγRI-positive monocytes were detected, and the numbers were comparable between ALS and control tissue.

When major injury is inflicted on the CNS, as in acute experimental allergic encephalitis,[39,40] a monocytic infiltrate is readily observed. When the injury is comparatively mild or restricted, for example, when the facial nerve is severed, no monocytic infiltration occurs. Only a microglial reaction is observed in the region of the facial nucleus.[41] Thus, the degree of monocytic infiltration may parallel the severity of immediate CNS damage. More chronic lesions, as in ALS, may involve no monocytic infiltration or a rate of infiltration too low to be detected on tissue sections.

The obvious targets for the upregulated levels of complement receptors on reactive microglia were tissues positive for C3d and C4d. These included many oligodendroglia,[36] in addition to damaged neurites. C3d and C4d are particularly significant complement components to identify in tissue. They are residual forms of C3 and C4 that have become attached by an ester or amide bond to tissue near the site of initiation of the complement cascade. C4d is associated with the classical pathway,

Figure 8. *Immunostaining for leukocytes. ALS precentral gyrus gray* (A) *and white* (B) *matter stained for T-helper/inducer (CD4+) lymphocytes.* C, D: *Nearby sections to A, B stained for T-cytotoxic/suppressor (CD8+) lymphocytes. Round cells can be seen in capillaries, with some invading the matrix. T8 cells considerably outnumber T4 cells. The field in* (D) *shows a substantial accumulation of CD8 positive lymphocytes in a small venule. Such accumulations were visible in many fields,* (A) *and* (B) *were fresh frozen, cryostat cut tissues, which produce somewhat inferior immunostaining;* (E, F) *control precentral gyrus gray matter immunostained for CD4* (E) *and CD8* (F) *lymphocytes. Neither class of lymphocyte appeared. Sections in* (A–E) *were doubly immunostained for T-lymphocytes and collagen type IV to illustrate the location of the capillaries.* G, H: *Peripheral lymph node stained for CD4 (*G, *NUTH/1 antibody) and CD8* (H). *The tissue was fixed similarly to brain tissue. Such fixation produces poor quality CD4 staining with the NUTH/1 antibody and no staining with the T4 antibody. The numbers of CD4 and CD8 positive lymphocytes are comparable.* I, J: *Nearby sections of the same peripheral lymph node stained for LCA* (I) *and FcγRI* (J). *Note the large number of LCA positive leukocytes and the few FcγRI monocytes. All photomicrographs are at the same magnification. Bar in* (J) = 100 μm.

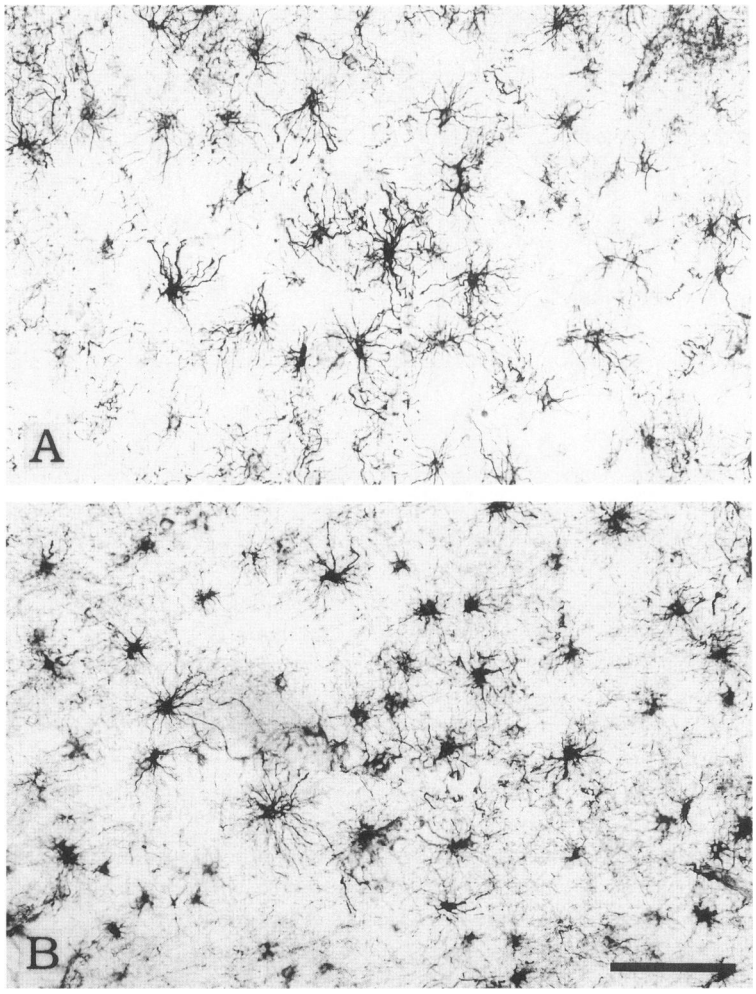

Figure 9. *Immunostaining of ALS precentral gyrus for GFAP.* **A**: *Gray matter. Notice the many intensely staining reactive astrocytes. Occasionally, these were grouped in clusters, particularly in layers II and III.* **B**: *White matter subjacent to* **(A)**. *Again, notice the many reactive astrocytes;* **(A, B)** *are at the same magnification; bar in* **(B)** = *100 μm.*

whereas C3d is associated with both the classical and alternative pathways. Fraction Bb of factor B and properdin (factor P) are associated only with the alternative pathway.[31,34] In normal tissue, C4d and C3d were detected only in residual serum of vessels, presumably as components of their parent proteins. In ALS-affected tissue, they were detected on complement-activated oligodendroglia and processes of degenerating neurons. No staining was obtained for fraction Bb of factor B or properdin (Figure 6G, H). Since there was a concordance between C3d and C4d staining, and an absence of staining for fraction Bb of factor B and factor P, it can be concluded that it is the classical, and not the alternative, pathway that is being activated in ALS.

The activators of the classical complement pathway in ALS are uncertain. They may be antibodies attached to specific antigens in tissues. Abnormal anti-ganglioside IgM antibodies in some sera of motor neuron disease (ALS) have been reported.[11–20] But the presence of antiganglioside antibodies is not a finding specific for ALS. Antibodies could result from a secondary response to motoneurons damaged by some other process. IgG has recently been reported histochemically to accumulate in upper and lower motoneurons of ALS.[21] Although we have found prominent staining for IgG receptors on resting microglia and prominent staining on reactive microglia, we have not, with the techniques so far employed, been able to detect consistently Ig molecules in tissue by immunocytochemistry. It would be anticipated, however, that such detection would be much more difficult than with complement proteins such as C3d and C4d because the latter are amplified products, in addition to being chemically attached to tissue. It has been shown that serum complement can be activated by cultured oligodendrocytes in the absence of antibody initiation, so it is possible that some other mechanism is responsible for complement fixation in ALS.[42]

The pattern of staining for ICAM-1, the ligand for the β-2 integrin LFA-1, was different from that for C3d and C4d (Figures 7, 8). Prominent staining of capillaries in the normal state suggests that this molecule is ordinarily produced by endothelial cells. In ALS-affected tissue, it was

patchily distributed in the tissue matrix, outlining in rare situations glial cells that could not be positively identified. The significance of this is yet to be determined.

MHC molecules serve as the restriction elements for T lymphocytes. T-cytotoxic/suppressor lymphocytes (CD8+) can interact only with cells bearing MHC class I (HLA-A,B,C) molecules, whereas T-helper/inducer lymphocytes (CD4+) can interact only with cells bearing MHC class II (HLA-D) molecules.[31] We have previously reported that reactive microglia simultaneously express both class I and class II MHC molecules in both rats[43] and humans.[44] In rats, MHC class I molecules appear to be more vigorously expressed than class II, whereas the reverse is true in humans. Endothelial cells in both species strongly express class I molecules so that capillaries in normal and pathologic tissues are strongly stained by class I antibodies.

In this study, capillaries of normal and ALS tissue were primarily stained by the HLA-A,B,C antibodies. Reactive microglia were additionally stained in ALS tissue when the DAB detection mixture was strengthened. By comparison with capillaries, however, the staining was weak.

In normal tissue, and in unaffected areas of ALS tissue, only a few microglia, mostly in white matter, were stained with antibodies to group II MHC glycoproteins. In affected ALS tissue, reactive microglia were prominently stained for HLA-DR, HLA-DP, and HLA-DQ. The contrast between affected and unaffected areas in ALS was so strong that regions of pathology could readily be detected with the naked eye on sections stained with antibodies to any of these antigens. The low levels of the group II glycoproteins on microglia in normal tissue and the high levels in diseased tissue[23] make these markers more revealing of pathology than other microglial markers such as LCA, FcγR1, or β-2 integrins.

The presence of T lymphocytes of both the helper/inducer and cytotoxic/suppressor subtypes was observed along capillary walls and in the tissue matrix in affected, but not in unaffected regions of ALS and not in any areas of controls. Lymphocytic cuffing around larger vessels, which is regarded classically as the hallmark of chronic inflammation in brain, was rare in ALS, but this may not be the most critical indicator of lymphocytic involvement. Lymphocytes in such cuffs are not positioned to interact with tissue cells. Their presence along capillaries and in the matrix may be a far more significant index of an immune system response. However, it is almost impossible to detect such lymphocytes in formalin-fixed paraffin-embedded tissue because their nuclei have a similar appearance to endogenous glial cells. Nevertheless, they can readily be detected immunohistochemically in carefully prepared tissue on the basis of their distinctive surface proteins.

Astrocytes positive for GFAP did not express class I or class II MHC glycoproteins, were not decorated with complement proteins, and showed no evidence of degeneration. Thus, the suggestion of Troost et al[24] that astrocytes might be responsible for a honeycomb pattern of HLA-A,B,C staining and thus the focus of immune attack could not be confirmed. Only reactive microglia widely expressed this complex. Additionally, rare glial cells of indeterminate identification did express MHC class I antigens strongly, but the significance of this remains to be determined.

The development of inflammatory changes in microglia, coupled with accumulations of T lymphocytes, favors either an autoimmune or slow virus theory of ALS. However, proof will require identification of either specific antigens in all cases or distinct viruses. The changes observed may be only secondary to some nonimmune process. The involvement of oligodendroglia adds a significant dimension because it indicates that the disease process is not restricted to neurons.

The techniques described here might be of immediate practical value. They are highly sensitive for reactive microglia and could be applied on a routine basis for greater accuracy in pathologic diagnosis.

Acknowledgments

The authors thank Dr. E. G. McGeer, Dr. A. Eisen, Dr. S.-G. Zhu, and Ms. Joane Sunahara for helpful discussions and technical assistance.

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