Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 10183–10187, November 1993
Neurobiology

Lymphocytes and macrophages outnumber oligodendroglia in normal fish spinal cord

(CD45/central nervous system/microglia/myelin turnover/autoimmunity)

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Communicated by Avrion Mitchison, July 15, 1993

ABSTRACT **As shown by staining with a monoclonal antibody against fish CD45, leukocytes are present in very large numbers in the fish central nervous system. Their subtypes were distinguished by electron microscopy and found to include all major hematogenous forms except thrombocytes, the most numerous being tissue macrophages and lymphocytes. As a population, they differ fundamentally from ramified microglia, the restricted form of myeloid cells present in the central nervous system in mammals. They are rare in most grey matter regions but are concentrated in myelinated fiber tracts as well as in certain strata of the radial glial network. The macrophages engulf discarded myelin and outnumber the oligodendrocytes in normal spinal cord white matter, where the density of lymphocytes is >5000-fold greater than reported in rat.**

At what stage in evolution did the central nervous system (CNS) come to exclude most circulating leukocytes (1, 2) and harbor its own characteristic network of resident myeloid cells (3, 4), the ramified microglia? The answer could cast light on various problems connected with the precarious isolation of the CNS behind the blood–brain barrier in mammals—for example, its susceptibility to immunologically mediated demyelinating diseases (5) and its resistance to neural repair (6, 7).

Although activated T lymphoblasts readily cross the blood–brain barrier (8), lymphocytes are normally almost undetectable within the parenchyma of the mammalian CNS (1). Macrophages are restricted to the meninges and perivascular spaces (3, 9), whereas granulocytes (4) and dendritic cells (10) appear to be excluded altogether. In contrast, microglia comprise up to 15% of all cells in various regions. They originate from monocytes that infiltrate the CNS in the neonate but express certain macrophage antigens at only very low levels and differ from other macrophages in two further important ways (4). (*i*) They are unusually highly ramified and distributed in regular mosaics, particularly in grey matter. (*ii*) They are an extremely stable resident population, resisting irradiation and donor replacement in bone marrow-chimeric animals (11).

Little is known about microglia in cold-blooded vertebrates (12–14), animals capable of CNS repair, and nothing at all is known on whether the CNS vascular endothelium restricts leukocyte traffic, as in mammals (15). In mammals, astrocytes induce the blood–brain barrier (16), and its properties may well differ in lower vertebrates, which retain radial glia in the adult.

Using the monoclonal antibody (mAb) FL.1, which reacts with leukocytes in certain fish, we recently found that these cells are present in the CNS in unexpectedly large numbers and varied forms (17). The transmembrane protein recognized by FL.1 (17) proved to be homologous to CD45, the leukocyte-common antigen in mammals (reviewed in ref. 18), as shown by its protein-tyrosine phosphatase activity and characteristic molecular weight isoforms (19).

This suggested that a wider variety of leukocytes may be present in the fish CNS than in mammals. Lacking reliable markers to define their subtypes, we decided to identify them as far as possible by electron microscopy (EM). We report here that the major forms of leukocytes found in the blood and/or hemopoietic organs are also normally present in the fish brain and spinal cord, particularly macrophages and lymphocytes, which are concentrated in white matter.

MATERIALS AND METHODS

Animals. The fish were 10- to 15-g adult *Oreochromis mossambicus* (20), an African species of cichlid (Perciform) fish, obtained as juveniles from the Institute of Aquaculture, Stirling University, Scotland, and reared in good health as judged by rapid growth and breeding.

Immunohistochemistry. Immunofluorescence microscopy was performed on frozen sections of paraformaldehyde-fixed material as described (17), using the mouse mAb FL.1 (17, 19) and a rabbit antiserum against carp apolipoprotein A-I (21). Fluorescent second antibodies were from Amersham International, and the sections were counterstained with Hoechst 33258 or propidium iodide. Thick (100 μm) sections were allowed to react with FL.1 in the presence of 0.03% Triton X-100 and then with gold-labeled second antibodies (Sigma; 5-nm particles) for up to 3 days each before preparation for EM.

EM. Samples were immersion-fixed in 150 mM sodium cacodylate-buffered 1% glutaraldehyde at pH 7.3 for 30 min and treated with buffered 1% OsO_4 for 1–2 hr before embedding in Epon. Sections were mounted on slot grids and stained with uranium and lead. Various somatic tissues (skin, intestine, gills), the blood, the hemopoietic organs (thymus, pronephros, spleen), and the CNS and peripheral nerves were surveyed. To map leukocyte distributions, cell profiles containing nuclei were identified at magnifications up to ×100,000, and their locations were entered on light micrographs of adjacent semithin sections prepared beforehand.

RESULTS AND DISCUSSION

Immunohistochemical Staining of Fish Leukocytes. Consistent with its specificity for fish CD45 (18), mAb FL.1 stains a wide range of leukocytes in different tissues in *Oreochromis*

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Abbreviations: CNS, central nervous system; PNS, peripheral nervous system; mAb, monoclonal antibody; EM, electron microscope (microscopy); SM, stratum marginale of the optic tectum; SO, stratum opticum; NP, tectal neuropil; SPV, stratum periventriculare; SE, subependyma.

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(17), including numerous nonramified forms within the CNS. These differ significantly in appearance and distribution from microglia in mammals. First, they are a heterogeneous population, comprising numerous tiny compact lymphocyte-like cells as well as extended amoeboid forms (Fig. 1). Second, even the most extended FL.1-positive (FL.1$^+$) cells are less ramified than quiescent microglia in mammals (Fig. 2*A*; compare figure 1 in ref. 4). They resemble more the simple microglia found in mammalian circumventricular organs, which are exposed to plasma proteins (4). However, though we noted that the fish CNS vascular endothelial cells are unfenestrated and possess tight junctions, the functional status of the blood–brain barrier in teleost fish is unknown. Finally, the FL.1$^+$ cells lack any regular mosaic distribution (Fig. 2*A*) and are sharply concentrated in certain regions such as the subependyma and white matter (discussed below). Whereas the uniquely ramified structure of mammalian microglia suggests that they function as a static network (4), the spectrum of FL.1$^+$ leukocytes in the fish CNS is in general very similar to that in various somatic tissues—e.g., the gut and skin.

Varied Hematogenous Cells in the Fish CNS. Using immunogold EM in the spinal cord and elsewhere, we confirmed that mAb FL.1 stains leukocytes exclusively (Fig. 2*B*); neither astrocytes (Fig. 2*B*) nor oligodendrocytes (Fig. 2*C*), identified by criteria given in the legend to Fig. 2, were labeled. By EM, the majority of leukocytes in the spinal cord and optic tectum fell into two categories, by criteria applied in both mammals (24) and fish (25). (*i*) Numerous small cells with attenuated dark cytoplasm, containing little but packed ribosomes, were identified as lymphocytes (Fig. 3*A*). They closely resembled lymphocytes in the blood (Fig. 3*D*), cerebrospinal fluid, and thymus, as distinct from granular leukocytes of the null (NK) cell type (24), and their presence in the fish CNS agrees with the earlier identification of small leukocytes in carp brain using mAbs raised against carp thymocytes (26). (*ii*) Numerous distinctly larger cells (Fig. 3*B*) were identified as tissue macrophages by their pale cytoplasm, occasional phagosomes (see below), and varied organelles, similar to those in macrophages found engulfing erythrocytes in the pronephros, a major lymphomyeloid tissue in fish.

Both of these types of cells were labeled by FL.1 immunogold, indicating that they correspond to the compact and amoeboid types of FL.1$^+$ cells seen by light microscopy (Fig. 2*A*), although the compact category includes a separate leukocyte described below. Moreover, an antiserum against carp apolipoprotein A-I (21), which selectively stains fish macrophages, distinguished the larger FL.1$^+$ amoeboid cells from the compact lymphoid forms in the majority of cases (data not shown).

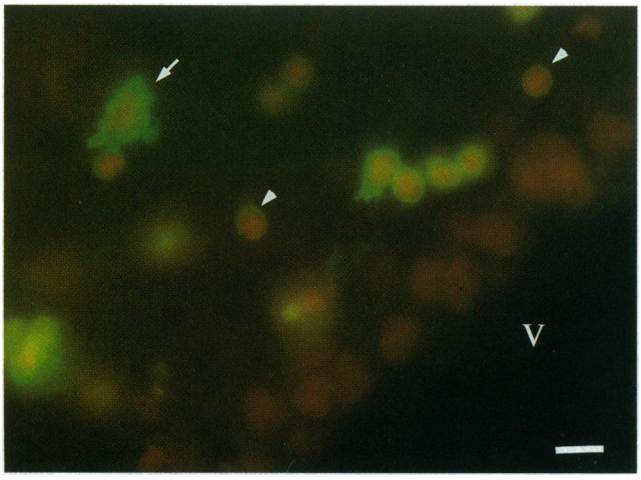

FIG. 1. Staining with mAb FL.1 (fluorescein isothiocyanate label) shows there are numerous compact lymphocytes (arrowheads) as well as larger amoeboid leukocytes (arrow) in the subependyma of the fish optic tectum. Counterstaining of the nuclei with propidium iodide shows the position of the ependymal cell layer lining the ventricle (V). (Bar = 5 μm.)

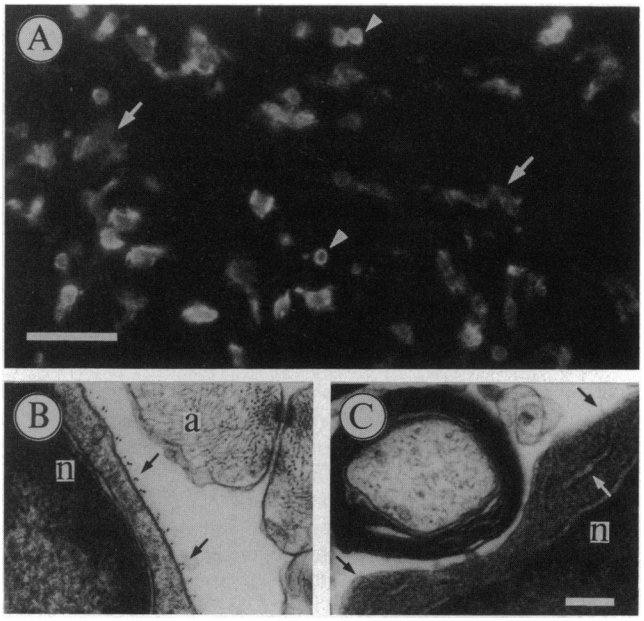

FIG. 2. Immunocytochemistry using mAb FL.1 in the spinal cord. (*A*) Immunofluorescence showing a high density of 3- to 4-μm (diameter) FL.1$^+$ leukocytes (arrowheads) as well as larger cells (arrows), which are stained comparatively weakly with FL.1. The difference of intensity of FL.1 staining (noted also using immunogold EM) would be consistent with CD45 being expressed at a much higher level in lymphocytes than in macrophages (22). (Bar = 20 μm.) (*B*) EM showing specific FL.1 immunogold labeling (arrows) of a spinal cord leukocyte, with characteristically patchy chromatin (compare Fig. 3*A*), compared with neighboring FL.1$^-$ astroglial processes (a) containing glial intermediate filaments. (*C*) Representative FL.1$^-$ (arrows) dark oligodendrocyte nearby in the same section as in *B*. Cells of the oligodendrocyte series (light to dark as in mammals) were readily distinguished from leukocytes by their stacked rough endoplasmic reticulum membranes (white arrow), more diffuse chromatin, and fine processes occasionally connected to myelin sheaths (see also refs. 17 and 23). In a sample of >300 cells in white matter, no labeling of oligodendrocytes or astroglia was detected; only labeling of varied leukocytes such as exemplified in Fig. 3 was observed. Labeled structures: a, astrocyte process; n, nucleus. (*A* and *B*, bar = 0.2 μm.)

Other leukocytes previously identified in fish (reviewed in ref. 27) were present in the CNS in very small numbers—namely, plasma cells and restricted forms of granulocytes. In addition, 5–20% of all FL.1$^+$ leukocytes in the optic tectum and spinal cord comprised a unique form of compact dark cells previously identified in fish pronephros, which we shall call Bielek cells (25). They resemble lymphocytes except for a characteristic rosette of pericentriolar vacuoles (Fig. 3*C*), somewhat resembling the "unzipped" form (28) of Langerhan's cell granules in mammals. Bielek cells are concentrated in the fish CNS: apart from rare examples in the thymus and pronephros (<<1/1000 cells), we found none in the other tissues examined (*Materials and Methods*), including the blood.

These three types of leukocytes account for much of the diversity of FL.1$^+$ cells (17) seen in the CNS by light microscopy. For comparison with mammals, we surveyed their numbers and distribution by EM in the spinal cord.

Density of Lymphoid Cells in *Oreochromis* Spinal Cord. In mammals, lymphocytes are so sparsely distributed in the

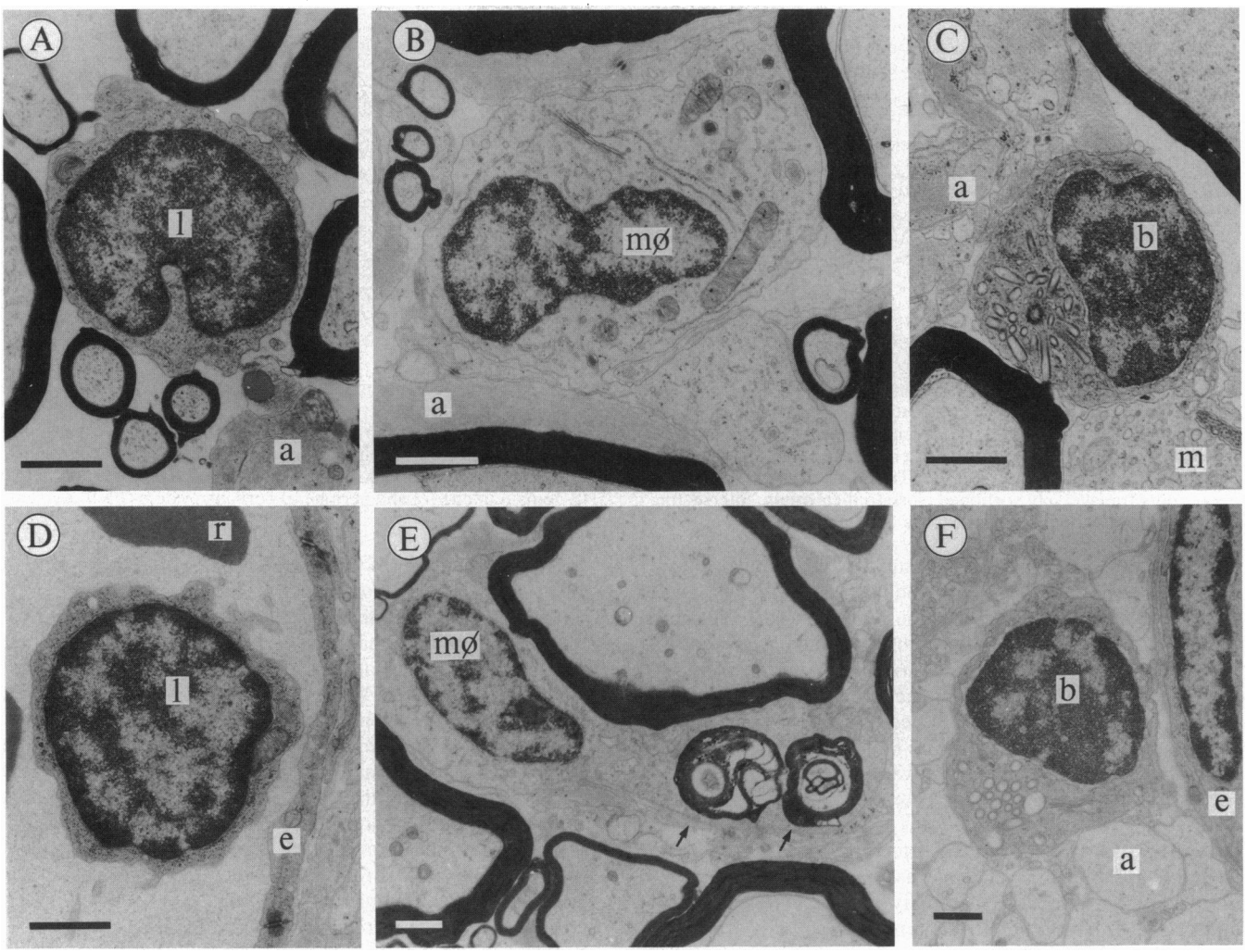

FIG. 3. EMs in spinal cord white matter (*A–E*, same material as analyzed in Fig. 4 *A* and *B*) and in the optic tectum (*F*) showing the principal forms of leukocytes identified in the fish CNS. Labeled structures: a and m, astrocyte and macrophage processes, respectively; e, vascular endothelium; r, red blood cell. (*A*) Lymphocyte (l), with patchy chromatin and attenuated cytoplasm containing little but ribosomes, as in blood lymphocytes (compare *D*). The CNS lymphocytes often showed lamellipodia, suggesting motility. (*B*) Tissue macrophage (mø), distinguished by the large amoeboid profile and light cytoplasm containing varied membrane organelles—e.g., vesicles, smooth endoplasmic reticulum, and phagosomes—as were also found in phagocytes in the pronephros. (*C*) Bielek cell (b), a small round form of leukocyte resembling a lymphocyte (compare *A* and *D*), but distinguished by the characteristic rod-shaped vacuoles surrounding the centriole (see also *F*). (*D*) Blood lymphocyte (l). (*E*) Macrophage (mø) showing engulfed myelin inclusions (arrowheads). Very similar preengulfed structures, with stacked membranes and granular material, were also found attached to myelin sheaths as well as apparently freed from them. (*F*) Bielek cell in the stratum marginale of the optic tectum (SM), with a corona of radial glial processes (a), such as surround capillaries (right). (Bars = 1 μm.)

CNS that they are difficult to detect immunocytochemically, although it is known that activated lymphoblastic T cells cross the blood–brain barrier readily. A recent immunocytochemical study (1) in rat spinal cord showed lymphocyte densities of about 0.04 per mm^2 in 5-μm sections from normal animals—that is, about 8 per mm^3. To provide a comparable estimate in fish, we mapped the positions of all leukocytes in transverse EM sections of the anterior spinal cord (*Materials and Methods*). Lymphocytes were distinguished as far as possible from Bielek cells (see Fig. 3), and the distributions of both are given in Fig. 4*A*, which is pooled from two sections separated by 20 μm. To standardize for varied cell sizes, only profiles containing nuclei were recorded: in white matter, the nuclei are elongated up to 5 μm axially, so the cell densities recorded are directly comparable, discounting shrinkage, to those of lymphocytes in rat spinal cord (1), and they are representative of numerous fish preparations. The lymphocytes were concentrated in white matter (thin outlines), but their density across the cord as a whole in Fig. 4*A* is 270 per mm^2, or about 5×10^4 per mm^3—i.e., >5000 times that reported in the rat. They were disseminated among the nerve fibers as much as near capillaries, and there were no signs of encephalitic attack—e.g., myelin sheath infiltration (29). Even Bielek cells were present at a density of 2×10^4 per mm^3.

The rat lymphocytes (1) were assayed by markers, such as CD8, expressed by subsets of lymphocytes, and could be an underestimate on this account. For example, $CD8^+$ cells comprised only 30% of all $CD45^+$ α,β-TCR^+ infiltrating lymphocytes recovered by fluorescence-activated cell sorter analysis from the brain in bone marrow-chimeras (22), but this is a small consideration besides the difference between fish and rat. The concentration of lymphocytes in fish blood has not been determined, but as a guide it is 3×10^3 per μl in humans (30), meaning that fish lymphocytes can probably be regarded as concentrated in the spinal cord, particularly in the white matter.

White Matter Macrophages in the Spinal Cord. In the spinal cord, macrophages were even more numerous than lymphocytes. Their density in Fig. 4*B* is 511 per mm^2, or 10^5 per mm^3,

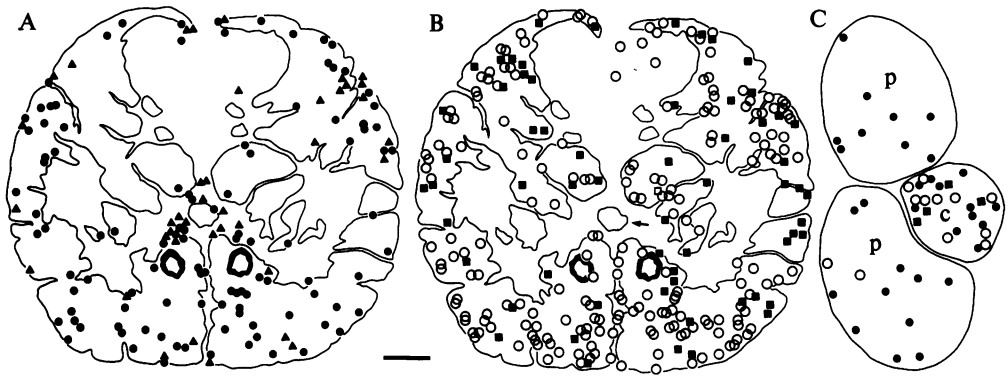

FIG. 4. Distribution of leukocytes in transverse EM sections of the spinal cord (*A* and *B*) and cranial roots (*C*). Thin outlines show the white matter, except in the case of the central canal, indicated by an arrow in *B*. ●, Lymphocytes; ▲, Bielek cells; ○, tissue macrophages; ■, oligodendrocytes. Only cell profiles containing nuclei were scored, and the diagrams are pooled in each case from two ultrathin sections separated by more than one cell length. The nuclei average about 5 μm in length (see text). (*A*) Spinal cord lymphocytes and Bielek cells. The two irregular black circles are Mauthner fibers. (*B*) Tissue macrophages and oligodendrocytes in the same sections as in *A*. (*C*) The three outlines are cranial nerve roots transversely sectioned close to their origin, intersecting the CNS domain in one (c) and peripheral nervous system (PNS) domains in the others (p), where the numerous Schwann cells are omitted for clarity. (Bar = 100 μm.)

but by far the majority were located in white matter (thin outlines), outnumbering the oligodendrocytes (density, 161 per mm^2) about 3-fold. This distribution differs radically from that of microglia in mammals, which form only 5% of cells in mouse corpus callosum (4) and are most numerous in grey matter regions of recent phylogenetic origin. Their semiregular 50- to 75-μm spacing in the neocortex gives a density of 10^4 per mm^3 or less, whereas the highest density of macrophages found in fish spinal cord white matter, in the ventral funiculi, was at least 20× greater at 2×10^5 per mm^3. Leukocytes and oligodendrocytes account for virtually all cells in fish spinal cord white matter, with only 3% made up by unidentifiable cases and rare astroglia.

What is the function of so many macrophages in normal adult fish white matter? In mammals, such cells appear in premyelinated fiber tracts in the neonate but disperse (it is thought) to form the microglia in the adult (4). By EM, about 1 in 20 of the fish macrophage profiles contained characteristic myelin phagosomes (Fig. 3*E*), suggesting that they function in the turnover and/or remodeling of myelin sheaths. Similar myelin bodies are continually shed from CNS paranodes (31, 32), and in fish spinal cord these structures, like the macrophages (Fig. 4*B*), occur most frequently among the large fibers in the ventral funiculi, thus showing the size association noted in mammals (31).

The 3-fold excess of macrophages over oligodendroglia seems generous for housekeeping, and to assess this further we also examined the fish PNS. Mammalian Schwann cells are considered self-sufficient in normally endocytosing their own myelin (32), although macrophages are summoned to deal with PNS Wallerian debris (4). In agreement, we found that myelinating Schwann cells in fish contain frequent myelin phagosomes, similar to those in the CNS macrophages, and that macrophages are rare in the PNS compared to the CNS, as shown at PNS/CNS boundaries in the cranial nerve roots (Fig. 4*C*).

Contrasting with the CNS macrophages in fish, microglia in mammals form only a small fraction (5–15%, ref. 4) of cells present in white matter. The composition of the macroglia also differs greatly. Whereas the astroglial network in fish white matter is formed by ascending radial glial processes, dedicated fibrous astrocytes reside within mammalian CNS fiber tracts (33), in similar numbers to oligodendroglia. There are hints that these astrocytes constitute an alternative myelin processing pathway. In mammals, engulfed myelin bodies reportedly appear mainly in astrocytic processes, and only rarely in microglia (32), whereas in normal fish we found them in macrophages only.

Regional Patterns in the Brain. Leukocytes in the fish spinal cord were concentrated mainly in the fiber tracts, but their patterns in the brain are more complex, indicating further selective associations. We examined these in the optic tectum (Fig. 5), whose exquisite vertical layering (described in ref. 34) dissociates cell populations that overlap in the spinal cord. In all cases we found that the leukocytes are associated with glial structures rather than with neuronal structures such as somas, dendrites, and synapses.

First, macrophages and lymphocytes were concentrated in the SO, the tectal afferent fiber layer, thus confirming their selective association with myelin (Fig. 5). However, Bielek cells were absent from the SO, indicating that their superficial distribution in the spinal cord (Fig. 4*A*) is unrelated to myelin *per se*. Instead, they were concentrated in the SM, but occluded from the neuropil by glial end feet, like those surrounding capillaries (Fig. 3*F*).

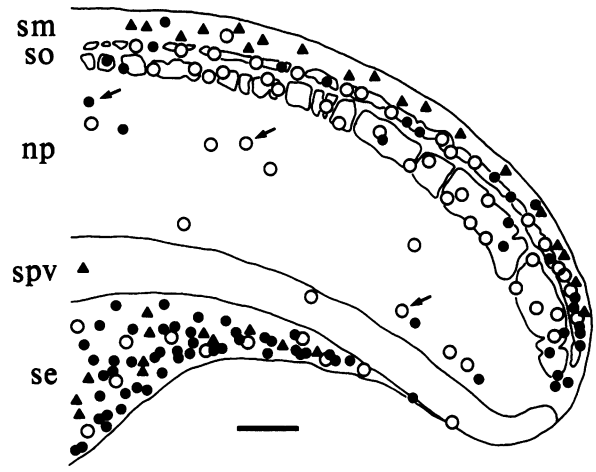

FIG. 5. Distribution of leukocytes identified in coronal EM sections (pooled from two sections as in Fig. 3) of the optic tectum at its lateral margin. ●, Lymphocytes; ○, tissue macrophages; ▲, Bielek cells; SO, stratum opticum, the outlines indicating fascicles of myelinated fibers; NP, synaptic neuropil containing dendrites of neurones located in the SPV, the stratum periventriculare; SE, subependyma. In the grey matter between the SPV and the SO, all the leukocytes shown bar three (arrowheads) were associated with scattered myelinated fiber bundles or capillaries. (Bar = 100 μm.)

Second, the subependymal plate (SE) of the fish optic tectum (Fig. 5) is an exclusively glial plexus, formed by processes of ependymal tanycytes and radial glia (35), and contains such a high concentration of leukocytes (Fig. 5) that it could be regarded as a lymphomyeloid tissue in its own right. It contains lymphocytes mainly, Bielek cells, and some macrophages, as well as rare plasma cells and granulocytes.

By contrast, leukocytes were seldom seen among the neuronal somas in the SPV or in the overlying NP, except where associated with capillaries and scattered myelinated fiber bundles (Fig. 5). This represents a major difference from mammals, where ramified microglia are present in large numbers in grey matter, suggesting neuronal-support functions (3).

Presumably quite different mechanisms account for the localization of the varied leukocytes in these three strata of the fish optic tectum. As in other CNS fiber tracts, the macrophages in the SO engulf myelin, but the function of the numerous lymphocytes associated with them is unknown. Unlike neurones (36), glia can express major histocompatibility antigens (37, 38), and the association of lymphocytes and Bielek cells with radial glial processes in the SE and the SM could signify immune-mediating functions.

Conclusions. Numerous lymphocytes and macrophages, resembling those in somatic tissues, are present in the CNS in fish, associated with glial structures, not neurones. They are clustered in defined strata of the radial glial palisade and also concentrated in white matter, where the macrophages engulf discarded myelin and can vigorously extend this normal activity during repair of the CNS (17). These fundamental differences may indicate the basis from which microglia in higher vertebrates evolved, and it is now important to determine whether, as we suspect, the fish CNS leukocytes are a renewable population capable of exchange with blood-borne cells.

This would cast new light on the characteristic seclusion of the CNS from circulating leukocytes in mammals, which possess hemopoietic germinal tissues (bone marrow and lymph nodes) lacking in fish (39). In mammals, myelin housekeeping is mediated by astrocytes and microglia (31, 32), long-term resident cells of the CNS that are relatively inefficient, compared to hematogenous macrophages, in supporting neural repair (4, 6, 7). Perhaps, with the evolution of mechanisms for affinity maturation of antibodies in higher vertebrates (40), central myelin turnover via macrophages in the presence of lymphocytes, as in fish, came to entail new hazards outweighing the penalties of injury to the CNS.

We thank M. C. Raff for helpful criticisms of an earlier version of the manuscript. Our thanks also go to Kate Kirwan and Susan Kruse for help with the figures and to Michal Schwartz for the gift of antiserum against fish apolipoprotein A-I.

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