THE NATURE OF THE DIVIDING CELLS AROUND AXOTOMIZED HYPOGLOSSAL NEURONES

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ABSTRACT

The origin of cells which appear in injured hypoglossal nuclei after hypoglossal axotomy was sought by carbon labelling and autoradiographic labelling experiments, and by electron microscopy. They do not enter the injured nucleus from the blood, nor do they reach the medulla by travelling up the severed nerve from the proximal stump, as shown by the carbon-labelling experiments. Autoradiography failed to reveal their origin. Electron microscopy showed that they are unlikely to be derived from pericytes in the injured nucleus. The long cisternae of the endoplasmic reticulum resemble those of the "neuroglial third type" or "multipotential glia", as well as those of "classical microglia" impregnated with Hortega's silver carbonate method and examined in the electron microscope. At the time of axotomy they are most likely to be resident in the brain, but their embryological origin is not known, so that although they are probably either "classical microglia" or "neuroglial third type," it is not yet possible to decide which. It is proposed to continue calling them "microglia."

INTRODUCTION

The classical view of microglia is that they are mesodermal elements which invade the mammalian brain from the meninges at or near the time of birth (4, 5), after which they remain scattered in the central nervous system throughout life, forming a permanent source of macrophages responding to any injury in the central nervous system if it occurs, but being otherwise quiescent.

Recent experiments with adult mammals have suggested, however, that the macrophages responding to brain injury are mostly derived from blood monocytes at the time of injury (1, 2, 6, 8, 14), and that only one third or less of the cells responding to needle wounds are resident in the brain at the time of injury. Other evidence suggests that several types of cells, such as pericytes (9, 10), astrocytes (13), and the multipotential glia or neuroglial third type (19, 20, 21) can become phagocytic according to the type of injury, and that the phagocytic response is not confined to cells of mesodermal origin.

When the hypoglossal nucleus of the rat is injured by axotomy of the hypoglossal nerve, numerous cells in the injured nucleus take up $^{3}$H-thymidine from about 2 to 10 days after axotomy, with a peak uptake at 3 to 4 days (22). In autoradiographs of isolated cell preparations most of these dividing cells have been regarded as macrophages (23). Adrian and Smotherman (1), on the basis of their autoradiographical experiments, have suggested that they enter the brain from the blood at the time of injury.

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Two types of experiments, carbon labelling and autoradiography, were designed to test this hypothesis and to explore alternative sources of origin. The aim of the carbon-labelling experiments was to see whether labelled macrophages would be attracted to injured hypoglossal nuclei from the blood, the peritoneal cavity, the cerebral ventricles, the cut proximal stump of the hypoglossal nerve, or from brain tissue itself. It had previously been shown that macrophages containing carbon particles were not necessarily satiated but could engulf further material, particularly if the carbon was adequately opsonized (7). Cerebral needle wounds were compared with axotomies to check on the influence of different types of injury and on the efficacy of the methods. While these experiments were in progress, Stenwig (15) published carbon-labelling experiments showing results in agreement with the ones reported here.

For the autoradiographic labelling, donor macrophages were radioactively labelled with $^3$H-uridine or $^3$H-thymidine and injected into recipients with axotomies, to see whether they would be attracted to the injured site from the blood, or the cerebral ventricles, or from the peritoneal cavity.

Previous experiments (14) using radioactive donor cells had used bone marrow preparations in which there were several cell types besides macrophages. The experiments reported here aimed at a purer population of donor macrophages, and for this, peritoneal cultures were selected.

In addition, evidence was drawn from our ultrastructural studies, the quantitative results of which are reported elsewhere (17).

**Material and Methods**

Adult male albino rats aged three months at the time of experiment were used.

*Carbon Experiments*

For the carbon labelling experiments the following medium was incubated at 37°C for 30 min. before injection: 1 vol. colloidal carbon, to 1 vol. Medium 199 containing 10% fresh calf serum as an opsonin (7). At first the carbon used was Aquadag (dialyzed for 3 days in running water to remove the ammonia, and then suspended, 1 ml. in 100 ml. of 1% sq. gelatine), but later the Gunther Wagner Peikau C11/1431a was preferred, since the particle size is more uniform. The volumes injected under light ether anaesthesia were as follows: 0.2 ml. intravenously into the tail vein; 1 ml. intraperitoneally; 50 µl intraventricularly into the right lateral cerebral ventricle; 5 µl locally into the right cerebral hemisphere. The schedule of carbon experiments is shown in table I.

For the axotomies, the left hypoglossal nerve was transected at the point where it crossed the carotid bifurcation (22). The needle wounds were all made dorso-ventrally into the left cerebral hemisphere with a 21-gauge hypodermic needle. One group of rats bore no needle wounds or axotomies, and served as controls. A second group bore axotomies, a third group bore needle wounds, and a fourth group bore both axotomies and needle wounds. Injections were deliberately 24 hours or more before needle wounds to allow time for carbon clearance (7, 12, 16).

In addition to these four groups, a fifth group received intravenous injections prior to axotomy to ensure maximal labelling of macrophages, and in a sixth group the cut ends of the hypoglossal nerve at the time of axotomy were placed in a gelatine sponge soaked in carbon, which was left in situ postoperatively, to see whether labelled macrophages would travel up the cut nerve to the injured hypoglossal nucleus.
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Fixation was by perfusion of 10% buffered neutral formalin through the left cardiac ventricle under ether anaesthesia. The brain was removed, and a transverse slice containing the needle wound if present, and another transverse slice containing the hypoglossal nuclei in the medulla, were fixed by immersion for an additional 48 hours. Sixty \( \mu \)m frozen sections were stained with neutral red and mounted in DPX. The thickness was the maximum consistent with clarity, for speed of scanning for presence or absence of labelled cells. Liver and spleen were also removed and treated as above. Blood was smeared and stained as above, and for Group 5, bone marrow was also smeared. For the sixth group in which the carbon was in a gelatine sponge around the cut ends of the nerve, the proximal and distal portions of nerve were also fixed and sectioned and stained as above.

**Autoradiographical Experiments**

**Series 1:** The aim of this experiment was to see whether radioactively labelled donor macrophages injected intravenously would be attracted to an injured hypoglossal nucleus from the blood.

Three days before day 0, a group of donor rats received 10 ml. per rat of nutrient broth intraperitoneally to stimulate growth of peritoneal macrophages. The intended recipients were litter mates of the donors, and received left hypoglossal axotomies on day 0. From day 1 to day 10 \(^{3}H\)-uridine-labelled cultured macrophages were injected intravenously into the recipients (under light ether anaesthesia), and the recipients were fixed 24 hours after injection, by perfusing with Carnoy solution through the left cardiac ventricle as above. The medulla containing the hypoglossal nuclei, and liver, and spleen were excised and fixed by immersion for a further 48 hours. Two \( \mu \)m thick wax sections were cut, brought down to water and washed overnight, and coated at 45°C in Ilford G5 nuclear research emulsion by dipping. They were dried in a stream of air, and stored in light-tight boxes in the refrigerator for 6 weeks. After development for 4 min. in Kodak D19 developer, and fixation in diluted Kodafix, they were washed for 1 hour, stained with cresyl fast violet, and mounted in DPX. Blood and bone marrow were smeared on subbed slides and coated by a wire loop method (18), exposed for 4 weeks, and after processing as above, were stained with pyronin-methyl green.

Macrophage cultures were made from the donors by peritoneal washings with Medium 199 containing 5 units of heparin per ml.\(^{2}\) (24). 10 ml. per rat of washings were obtained, mixed with 10% human PO serum and incubated for 24 hours at 37°C. Each culture was then washed several times in Medium 199, and incubated for 1 hour at 37°C in 10 ml. Medium 199 containing 100 \( \mu \)C of \(^{3}H\)-uridine. After several washings in 199, the macrophages were scraped off the glass culture dish with a wooden spatula, centrifuged gently, the supernatant decanted, and the sediment suspended in 0.5 ml. of 199. A drop of the suspension was counted in a Neubauer chamber, and the remainder (7 to 11 million cells) injected into the tail vein of the recipient rat. The cells remaining in the glass culture dish were quantitatively analyzed to check the purity of the culture, and a sample coated for autoradiography to check on the labelling success. Viability was checked and found to be satisfactory by incubation of a sample of the culture for 2 hours at 37°C in Medium 199 containing carbon. This at the same time provided an additional check on the purity of the culture.

**Series 2:** The aim of this experiment was to see whether radioactive donor macrophages would be attracted to injured hypoglossal nuclei from the cerebral ventricles.

In this series the donor rats were also the recipients. Nembutal was used as the anaesthetic throughout this experiment. On day 0 they received left hypoglossal axotomies and intraperitoneal injections of 10 ml. per rat of nutrient broth. On day 3 cultures were set up as before, but labelled from the start with 10 \( \mu \)C \(^{3}H\)-thymidine per ml. of culture. After 24 hours the cultures were washed, scraped, centrifuged, the supernatant decanted, and the

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1 Stuart, A. E. Personal communication.
sediment suspended, and counted as before. The cells were suspended in artificial cerebrospinal fluid and each rat received its own macrophages back, injected into the cerebral ventricle this time (100 μl per rat, containing 1 to 11 million cells). As before the cultures were counted for purity, viability, and labelling success. At 1½ hours, 24 hours and 48 hours after injection, transverse slices of brain containing the injection site, and the hypoglossals were removed, also liver, spleen, blood, bone marrow, alveolar washings, and peritoneal smears. Autoradiography was similar to that for series 1.

Series 3: The aim of this experiment was to see whether radioactive cells would be attracted to injured hypoglossal or sciatic nuclei from the peritoneal cavity.

In series 3 donors and recipients belonged to the same inbred strain. Donors were injected with nutrient broth as for series 1 three days before day 0. On day 0 recipients received left hypoglossal and left sciatic transections. Each day up to day 8 peritoneal macrophages were harvested from a batch of donors, and tubes (one per donor) of cells injected with 10 μCi per ml of \(^{3}H\)-uridine, and incubated for 30 min. at 37°C. After several centrifugations and washings with Medium 199, the labelled cells were suspended in 2 ml. 199 per tube. Two donors (4 ml.) were pooled to inject one recipient. Counts and smears were made of the cells as before. Each recipient received 31 to 75 million cells intraperitoneally under light ether anaesthesia, and was fixed in Carnoy after 24 hours, removing the same tissues as in series 2, with also the portion of spinal cord containing the dorsal and ventral roots for the sciatic nerves.

For all series, normal rats were compared with those which had received operations, and they were injected with labelled cells just like operated rats.

**Electron Microscopy**

Normal rats and axotomized rats 1 day to 12 weeks after operation were fixed by double aldehyde perfusion, and the hypoglossal nuclei removed, post-fixed, and embedded in TAAB resin as described by Sumner and Sutherland (17). Sections were stained with uranyl acetate and lead citrate, and photographed with an AEI EM6B electron microscope (17).

**RESULTS**

**Carbon Experiments**

**Group 1 (controls with carbon injections only):** Fifteen days after intravenous injection of carbon, many labelled cells were seen in liver, but not in spleen, blood or brain. Fifteen days after 5 μl of carbon were injected locally in the brain, sparse labelled cells were seen in liver, but none in spleen or blood. Labelled cells were present in the brain at the site of injection.

**Group 2 (rats with left hypoglossal axotomies prior to carbon injections):** After intravenous injections, labelled cells were seen in liver and spleen to variable extents at 5 and 7 days. None were seen in blood, or brain. After local brain injections scarce labelled cells were seen in liver, but none elsewhere except at the site of injection where labelled cells were present. After intraperitoneal injections labelled cells were common in liver and spleen but none were seen elsewhere. After intraventricular injections scarce labelled cells were observed in spleen, but none were seen elsewhere except at the site of injection. Particles of carbon were seen still present in the cerebral ventricle clinging to ependymal cilia at the injection site, as well as present inside cells at that site.

**Group 3 (rats with needle wounds after carbon injections):** As with Group 2, carbon was seen in cells of liver and spleen to a variable extent after intrave-
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<td>Intravenous injection</td>
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<td>Group 3 (needle wounds)</td>
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<td>1 day</td>
<td>Intravenous injection</td>
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<td>needle wound</td>
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Op. = operation.
fig. 1. Needle wound in the brain of a rat 15 days after intravenous injection of carbon, 14 days after the needle wound. 60 µm frozen section stained with neutral red. × 500. B, brain tissue adjacent to wound; C, cells containing carbon particles; M, mast cells; W, wound filled with carbon-laden cells, many of which have also ingested erythrocytes.

ous injections, to a great extent after intraperitoneal injections, and scarcely after local injections into the brain. None was seen in the blood. All the needle wounds contained carbon-laden cells from 3 days onwards after the wound. The quantities of labelled cells were greatest after intravenous injections; at 14 days after the wound, 15 days after intravenous injection, most of the cells in the wound contained carbon (Fig. 1). After local brain injections some labelled cells remained at the injection site although some responded to the wound in the opposite hemisphere.

Group 4 (rats with axotomies prior to carbon injections, and needle wounds after the injections): Results for liver, spleen and blood were similar to those for the corresponding injections in Group 3. As with Group 3 also, labelled cells were spotted in the needle wound from 4 days onwards, but none were spotted in the hypoglossal nuclei after either intravenous or local brain injections.

Group 5 (rats with axotomies after repeated intravenous injections of carbon): Results for liver, spleen and blood were typical for intravenous injections (as Groups 2, 3, and 4), scarce carbon-laden cells were observed in bone marrow, but none were seen in the hypoglossal nuclei from 7 to 21 days after axotomy.

Group 6 (rats with carbon in sponge around cut ends of hypoglossal nerve): At 1 day after implantation of a carbon-soaked gelatine sponge, free carbon was observed in the connective tissue sheath clothing the nerve, and at the proximal tip of the cut nerve amongst the axons. Some cells among the cut axons and in the sheath contained intracellular carbon. No carbon was present
in the medulla. At 2 days, carbon particles among axons and inside cells were seen some distance from the tip of the proximal cut stump. At 4 days, all the carbon in the sheath was intracellular, and as at 2 days, a few labelled cells were seen among the cut axons in the proximal stump. Carbon particles were observed further up the axons of the proximal stump than at 2 days. No carbon was seen in the medulla. At 11 days carbon was still intracellular in the proximal stump. Laden cells, and carbon particles in axons, had travelled further up the nerve, but no carbon was present in the medulla, although the increase in numbers of cells in the injured hypoglossal nucleus was apparent.

**Autoradiographical Experiments**

**Series 1 (Labelled donor cells injected intravenously into litter mates of donors):** The cultures from the donors were 87 to 100% peritoneal macrophages, 0 to 6% lymphocytes, and 0 to 9% mast cells. The $^3$H-uridine labelled every cell heavily. In spite of this no labelled cells were observed in any of the tissues studied in the recipient rats, from 2 to 11 days after left hypoglossal axotomy.

**Series 2 (Recipients received intraventricular injections of their own labelled cells):** The cultures were composed as in Series 1. $^3$H-thymidine labelled 96.4 to 100% of the cells. No labelled cells were found in the recipient rats except for a few around the injection site at 4, 5 and 6 days after left hypoglossal axotomy.

**Series 3 (Labelled donor cells from same inbred strain as recipients, injected intraperitoneally):** 40 to 75% of the injected cells were labelled with $^3$H-uridine. No labelled cells were found in the recipients except for a few in the liver, from 2 to 9 days after left hypoglossal and left sciatic axotomy.

**Electron Microscopy**

Injured hypoglossal nuclei from 2 days to about 5 weeks after axotomy were characterized by the increase in numbers of a cell type which was absent or rare in normal nuclei. We have previously called them microglia (17). Their elongated or irregularly shaped nuclei contained peripheral lumps of electron dense chromatin, with elsewhere, granular chromatin. Their cytoplasm was homogenous and moderately electron dense, with few but prominent Golgi profiles, few dense bodies and mitochondria, and endoplasmic reticulum in the form of a few long twisting cisternae clothed at irregular intervals with ribosomes (Fig. 2). The cisternae extended along the cytoplasmic processes from the perinuclear zone. Inside the processes there were also channels which communicated with the exterior, and were often twisted and coiled at the ends of the processes. Among these channels were vesicles with or without electron dense contents (Fig. 3). The cytoplasmic processes wrapped around neurone perikarya and some dendrites.

Astrocytes and oligodendrocytes in the injured nuclei had an ultrastructural appearance similar to that in normal rats, and so also did pericytes. Pericytes lay between two layers of intact basal lamina both in normal and injured.
nuclei. Pericytes did not possess the characteristic long twisting cisternae of the endoplasmic reticulum, or the channels and vesicles shown by the putative microglia above. Their cytoplasm was granular and not so electron dense, and their rough endoplasmic reticulum was in the form of short cisternae. Golgi apparatus, dense bodies, and mitochondria were present but unremarkable.
Astrocytes were distinguished by their electron-lucent cytoplasm in which were scattered organelles. Those characteristic of astrocytes were glycogen particles, and bundles of fibrils. Their nuclei were homogeneously granular. Oligodendrocytes had electron-dense cytoplasm which was more granular than that of the possible microglia. Their endoplasmic reticulum was in the form of short cisternae and not in long twisting cisternae. Their nuclei were round or oval, with peripheral lumps of chromatin.

DISCUSSION

The results of the carbon labelling experiments show that whereas carbon-laden macrophages will enter a needle wound in the brain from the blood, no such blood-borne cells arrive in the injured hypoglossal nucleus after hypoglossal axotomy. These results agree with Stenwig's (15). Labelled cells did not travel up the injured nerve from the cut stump to the medulla either.

The scarcity of labelled cells seen in recipients after injection of radioactive
donor cells suggests that the label is quickly diluted either by mitosis, or by cell death, ingestion and mitosis. Roessman and Friede (14) only found cells in recipients after diligent search, although their donor cells had been heavily labelled.

If the cells which respond to axotomy are not systemic, then presumably they must be present in the brain already when the axotomy is performed. In normal rats, scarce cells of the type which increase in numbers after axotomy, were occasionally observed in the hypoglossal nucleus with the electron microscope. Whether these few are enough to produce the massive increase in numbers in so short a time is unknown. Migration of cells into the injured nucleus from other parts of the brain could be postulated. Injections of carbon locally into the brain did not produce labelled cells in the hypoglossal nucleus, but it could be argued that they were too remote from the injured nucleus. Even when an axotomized rat also possessed a needle wound, the enormous numbers of carbon-laden cells seen in and around the wound did not respond to the axotomy also.

Are the cells in the injured nucleus not macrophages at all? Electron microscopy gave no evidence of phagocytosis in these cells. Channels in their cytoplasm associated with vesicles in their processes, however, suggests pinocytosis, as mentioned elsewhere (17). The few long twisting cisternae of the endoplasmic reticulum were observed by Vaughn et al. (19), Vaughn and Pease (20), and Vaughn and Peters (21) in cells seen in optic nerve, derived embryologically from primitive ependyma. These they termed "neuroglial third type" or "multi-potential glial cells." They argued that they could not be classical microglia (4, 5) because of their ectodermal origin. Mori and Leblond (11) identified classical microglia by light microscopy with the Hortega silver carbonate method, and then examined them with the electron microscope. Their appearance was similar to the cells of Vaughn et al., complete with the long twisting cisternae, but, although some were interstitial in the neuropil, some were in the position of pericytes. In the injured hypoglossal nucleus there was no evidence that pericytes gave rise to the cells reported here, since the basal lamina remained intact. The long cisternae and channels were not present in hypoglossal pericytes.

Microglia observed by Blinzinger and Kreutzberg (3) possess the long cisternae and other similarities to the cell type reported here, such as the electron dense cytoplasm, and coarsely clumped peripheral nuclear chromatin.

Until the embryological origin of the cells which respond to hypoglossal axotomy is known, it is not possible to resolve the question of whether they are classical microglia or neuroglial third type, since ultrastructurally these two kinds of cell appear to be similar.

The putative microglia seen in injured hypoglossal nuclei with the electron microscope doubtless correspond to the cells reported to be "macrophages" by Watson (23) in 3H-thymidine autoradiographs of isolated cell preparations of injured hypoglossal nuclei. No other cell type is increasing so markedly in numbers at that time.
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In conclusion, it appears that after hypoglossal axotomy cells resident in the adult brain produce daughter cells in the injured nucleus which resemble ultrastructurally both classical microglia and neuroglial third type, and which disappear from the injured nucleus at about 5 weeks after axotomy. It is proposed to continue calling them "microglia", as we have done previously (17).

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