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Directional and compartmentalised drainage of interstitial fluid and cerebrospinal fluid from the rat brain*

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Summary. Pathways for drainage of interstitial fluid and cerebrospinal fluid from the rat brain were investigated by the injection of 2–5 μl Indian ink into cerebral white and grey matter and into the subarachnoid space over the vertex of the left frontal lobe. Animals were killed by formalin or glutaraldehyde perfusion 5 min–2 years after injection, and the distribution of ink over the surface of the brain, in 2-mm slices of brain cleared in cedar wood oil, in paraffin sections and by electron microscopy was documented. These investigations showed that carbon particles were distributed diffusely through the interstitial spaces of the white matter whereas they spread selectively along perivascular spaces in the grey matter outlining both arteries and veins and extending to surround capillaries within 1 h. Carbon particles were rapidly ingested by perivascular cells and, to some extent, by meningeal cells surrounding the larger vessels. Very little movement of carbon-labelled perivascular cells and perivascular macrophages was seen after 2 years. Carbon particles entering the subarachnoid space over the vertex of the cerebral hemispheres drained along selected paravascular and subfrontal pathways in the subarachnoid space to the cribriform plate and thence into nasal lymphatics and cervical lymph nodes. These studies demonstrate the diffuse spread of fluid-borne tracers through cerebral white matter in the rat, the perivascular spread of tracer in grey matter and the compartmentalised directional flow or tracer through the subarachnoid space to the cribriform plate and nasal lymphatics. Furthermore, particulate matter selectively injected into perivascular spaces in rat grey matter is rapidly and efficiently ingested by perivascular cells.

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The drainage of interstitial fluid and cerebrospinal fluid to cervical lymphatics and lymph nodes is well documented in a number of different mammalian species including the rabbit [2, 8], the cat [18] and the rat [7, 24]. Such drainage can be very rapid with tracers like Indian ink reaching cervical lymph nodes within a few seconds of injection into the cerebrospinal fluid [28]. Tracers and antigens injected into the grey matter of the rat brain also drain to cervical lymph nodes but at a slower rate [13, 24].

The results of previous studies using tracers such as Dextran blue 2000 [6], Indian ink [3] or horseradish peroxidase [22] suggest that drainage of interstitial fluid from the brain is along perivascular spaces, both in the grey matter and in the subarachnoid space [24]. Anatomical continuity of perivascular spaces in the cerebral cortex and vessels in the subarachnoid space has been demonstrated by ultrastructural studies in man [1, 14, 29] and rat [15].

Although, there is good evidence that interstitial fluid from the brain drains along perivascular spaces into the cerebrospinal fluid and subsequently into nasal lymphatics in several species, the exact anatomical pathways have still not been fully elucidated. The objects of the present study, therefore, are first to define the anatomical pathways for the drainage of interstitial fluid from the brain to nasal lymphatics in the rat, and second to investigate the efficiency of perivascular cells in clearing particulate tracer from perivascular drainage pathways. The pathway of drainage of cerebrospinal fluid from the subarachnoid space into nasal lymphatics is the subject of a further publication (Pantazis & Weller, in preparation).

Indian ink is used in this study as a tracer as carbon particles are poorly diffusable and are more likely to stay

in position once the pressure of the injection has subsided. Although Indian ink is not a physiological tracer, it is proposed that the initial drainage pathways can be demonstrated in this way without the complication of subsequent diffusion of tracer.

Materials and methods

A total of 41 young adult Wistar rats, approximately 150 g in weight and of both sexes, was used in this study. The animals were anaesthetised with ether and placed in a stereotactic frame. A 30-guage needle was inserted into the left cerebral hemisphere through a burr hole at one of two sites: (a) 2.5 mm lateral to the bregma to a depth of 5.5 mm, this site was calculated for injection into the caudo-putamen; (b) 4 mm lateral to the bregma and 4.5 mm in depth, these co-ordinates were calculated for injection into the white matter. Rotring Indian ink suspension (1 or 2 μl) were injected over a period of several minutes into the selected site using a micrometre attachment. The needle was then withdrawn, the burr hole closed with bone wax and the skin sutured. Care was taken to avoid contamination of extracranial tissues with Indian ink. In 7 animals, 0.5 μl sesame oil was drawn into the syringe both before and after the Indian ink. By injecting oil either side, Indian ink was prevented from entering the subarachnoid space over the surface of the hemisphere on entry and withdrawal of the needle.

Except for those animals killed within the 1st h after injection, all animals were allowed to recover from the anaesthetic and none showed any neurological deficit. Animals were subsequently anaesthetised and perfused with 10% buffered formalin (35 animals) or 3% glutaraldehyde in 0.1 M cacodylate buffer (4 animals) by intracardiac perfusion. Animals were killed at regular intervals between 5 min and 2 years after injection.

Following perfusion fixation, the brain was removed. Blocks of cribriform plate of the skull and nasal mucosa were taken together with deep and superficial cervical lymph nodes. Samples of liver, kidney, spleen, lung and lymph nodes from the para-aortic and inguinal regions were also taken for histology.

Two control animals were perfused through the left cardiac ventricle with salin; 10 ml of Indian ink in 10% gelatin were then injected to outline the vasculature of the brain. Following removal from the skull, the brains of these animals were fixed by immersion in 10% buffered formalin for 24 h.

The brains of the injected animals were prepared in one or more of the following ways. External surfaces of the cerebrum were carefully examined and the distribution of Indian ink in the subarachnoid space was documented. The brains of 15 rats perfused with formalin 1 h–2 years after the intracerebral injection of Indian ink were cut coronally into 2-mm slices which were then dehydrated in alcohol and cleared in cedar wood oil. Following clearing, the slices were transilluminated and either photographed or documented as camera-lucida drawings at ×20 magnification, constructed by the use of a Leitz drawing tube. Blocks of brain from animals injected systemically with Indian ink were prepared in a similar way. Samples of cleared brain tissue were taken and prepared for resin embedding to identify specific structures and the distribution of the Indian ink.

Coronal slices of cerebral hemispheres and systemic organs from formalin-fixed rats were dehydrated and embedded in paraffin. Paraffin sections (5 μm) were stained with haematoxylin and eosin (H&E), haematoxylin van Gieson (HvG), and the Gordon and Sweet reticulin technique; brain sections were also stained with the Klüver-Barrera technique. Specimens taken from the cribriform plate and nasal mucosa of rats were decalcified in nitric acid for 24 h and embedded in paraffin. Sections were stained with H&E, HvG and reticulin techniques.

Blocks of brain from Indian ink injected areas were taken from the four animals perfused with glutaraldehyde and from selected areas of formalin-fixed brain cleared in cedar wood oil, post-fixed in osmium tetroxide, dehydrated in alcohols and propylene oxide and embedded in Spurr resin. Sections (0.75–1 μm) were stained with toluidine blue and ultrathin sections were cut from defined regions, stained with lead citrate and examined in a Hitachi H7000 transmission electron microscope at 60 kV.

Results

Distribution of Indian ink injected into the rat cerebrum

Coronal slices of rat brain cleared in cedar wool oil allowed the distribution of Indian ink to be visualised and recorded by camera-lucida drawings in which several focal planes within the same 2-mm section could be included (Fig. 1). Figure 1a is a coronal slice of rat brain perfused with Indian ink gelatin and depicts the distribution and orientation of arteries (left of diagram) and veins (right of diagram). Only the major vessels are shown but it is clearly seen that the arteries branch obliquely whereas the tributaries of veins are perpendicular to the major venous channels.

In Fig. 1b, the camera-lucida drawings represent coronal slices of rat brain taken through the region of the optic chiasm; the drawings were originally constructed at ×20 magnification and then reduced to ×3 magnification for illustration. Figure 2 shows a photograph of a typical preparation from which the camera-lucida drawings were prepared. A brain taken 1 h after injection (Fig. 1b) shows a vertical injection tract from which ink has spread diffusely into white matter. By contrast, Indian ink injected into the grey matter has spread along the perivascular spaces to outline small vessels. Similar vessels are seen in the cortex. The injection in the 12-h animal was specifically aimed at the white matter and shows a large amount of ink spreading diffusely through the white matter; little has entered the perivascular spaces of vessels in the grey matter. Extensive perivascular spread of ink is seen in the grey matter of the cortex and the caudo-putamen in the 24-h specimen; there is also diffuse spread of ink in the white matter. Numerous small vessels are outlined and a major branch of the middle cerebral artery is also visualized by the ink. Although the same basic pattern of perivascular spread in the grey matter and diffuse spread in the white matter is seen at 6 weeks (Figs. 1b, 2) the profiles of the perivascular Indian ink and even the diffusely distributed Indian ink in the white matter have become fragmented due to ingestion of ink by macrophages. This picture has changed little in the 21-week and 6-month specimens, and the distinct pattern of diffuse white matter spread and perivascular spread of ink in the grey matter is maintained even 1 and 2 years after injection.

Figure 2 is a transillumination photograph of a cedar wood oil-cleared specimen 6 weeks after injection. It shows slightly less detail than the drawing in Fig. 1b as vessels out of the plane of focus in Fig. 2 have been included in Fig. 1b. Branches of the middle cerebral artery are outlined by black ink deposit as are vessels in

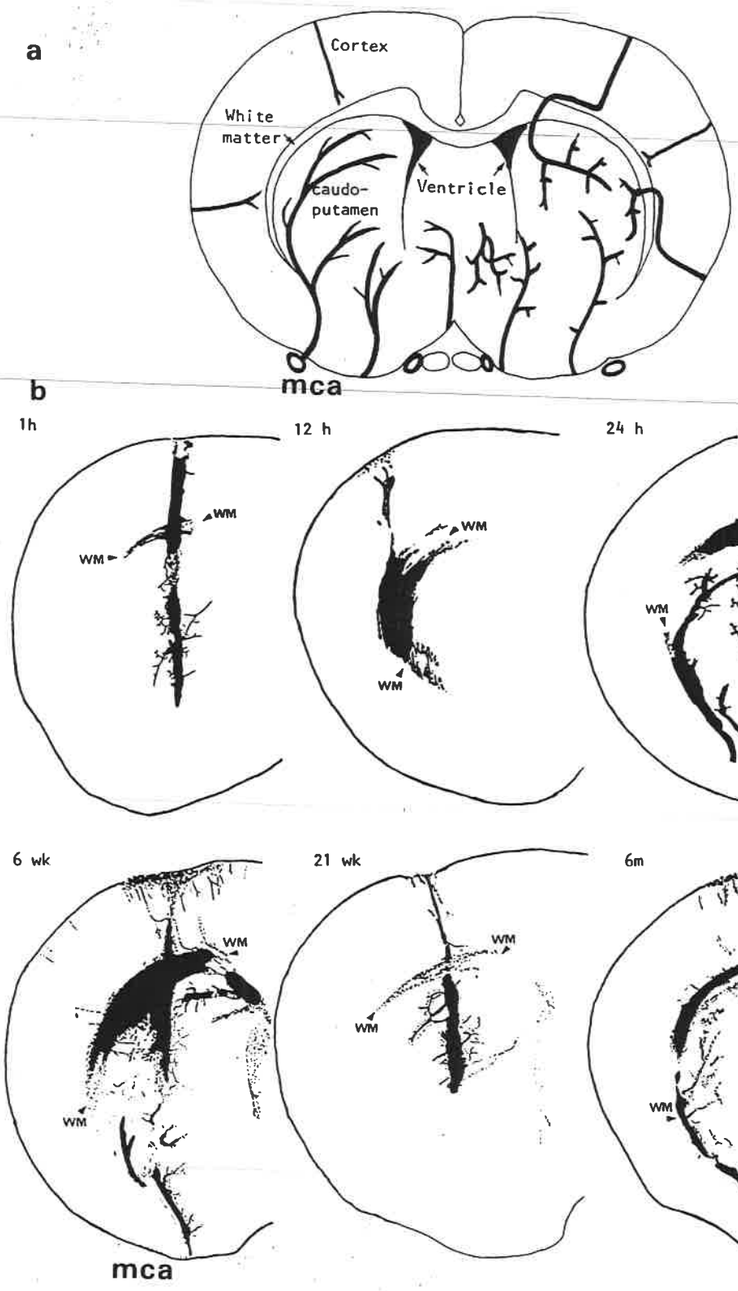
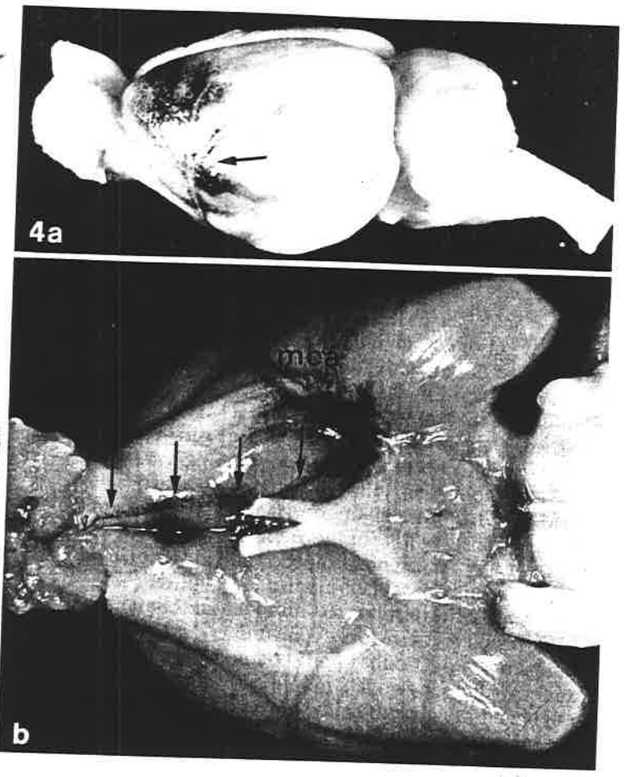
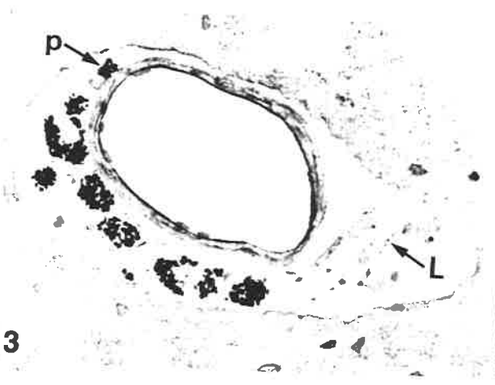


Fig. 1. **a** Camera-lucida drawing of a cedar wood oil-cleared section of rat brain showing the vasculature injected with Indian ink. Arteries are depicted on the *left* and veins are outlined on the *right*. *mca:* Middle cerebral artery. **b** Camera-lucida drawings of cedar wood oil-cleared 2-mm slices of rat brains injected with 1–2 µl Indian ink 1 h to 6 months previously. There is diffuse spread of ink through the white matter (*WM*), and perivascular spread in the grey matter. *mca:* Branches of middle cerebral artery





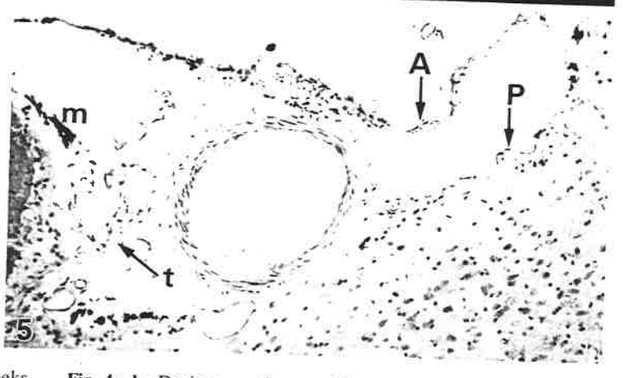


Fig. 2. Coronal slice of cedar wood oil-cleared rat brain 6 weeks after injection of Indian ink. There is diffuse spread of ink through the white matter (*wm*) and perivascular spread in central grey matter and cortex. Injection site (*arrow*). Branches of middle cerebral artery (*mca*). Some ink has ruptured into the ventricle (*V*)

Fig. 3. A branch of the middle cerebral artery in the caudo-putamen of Fig. 2 showing Indian ink particles in a perivascular cell attached to the vessel wall (*p*). in macrophages in the dilated perivascular space and in leptomeningeal cells (*L*). No free ink remains in the perivascular space. Toluidine blue-stained resin section. × 500

Fig. 4a, b. Drainage pathways of Indian ink in the subarachnoid space (4 weeks after injection). **a** Ink injected over the vertex of the hemisphere (*top*) is distributed in broad channels adjacent to branches of the middle cerebral artery (*arrow*). **b** At the base of the brain. ink outlines the middle cerebral artery (*mca*) and there is some pooling of ink at the circle of Willis. A distinct line of ink tracks forwards to the olfactory bulb (*arrows*)

Fig. 5. Indian ink in the subarachnoid space surrounding the middle cerebral artery (*centre*) 4 weeks after injection. Ink is within macrophages (*m*) in the subarachnoid space but not in the perivascular space immediately adjacent to the artery. Trabeculae (*t*) pass from the arachnoid (*A*) to the pia (*P*) forming channels within the subarachnoid space. H&E × 80

the cerebral cortex. Indian ink deposited on the surface of the brain at the time of injection has spread along the perivascular spaces of cortical arteries and veins; this was first seen 12–24 h after injection. It is not clear, however, whether the ink was deposited in the subarachnoid or the subpial space for this spread to occur.

Examination of paraffin sections and 0.75- to 1.0-μm resin sections confirmed the perivascular spread of Indian ink in the grey matter of the caudo-putamen and the cortex. Particles of ink were detected mainly around arteries and veins but small amounts were also seen around capillaries. No such selective perivascular spread was seen in the white matter; here the ink had spread diffusely between nerve fibres. By 1 week, much of the ink had been taken up by macrophages in the white matter and in the injection sites and by perivascular cells flattened against vessel walls in the grey matter. Figure 3 is a section taken through the branch of the middle cerebral artery of the specimen illustrated in Fig. 2. It shows a large artery surrounded by a dilated perivascular space. Particles of Indian ink are seen in a perivascular cell adhering to the outer aspect of the vessel and in globular macrophages lying free from the vessel but within the perivascular space. Small numbers of individual particles of Indian ink are also seen in stellate cells within the perivascular space which, by electron microscopy, resembled leptomeningeal cells. Few ink particles remained free in the perivascular space after 4–6 weeks.

Indian ink which had escaped from perivascular spaces into the brain parenchyma was ingested by microglia and by macrophages. Although the phagocytic cells containing Indian ink became clumped within perivascular spaces producing the punctate appearance seen in the 21-week and 6-month animals in Fig. 1b, it was not possible to detect any substantial movement of macrophages within perivascular spaces during the 2-year period of observation.

Dispersal of tracer in the subarachnoid space

In those animals in which a small amount of oil had been injected both before and after the Indian ink injection, no Indian ink leaked into the subarachnoid space and in these animals no Indian ink reached the cervical lymph nodes. For the majority of animals, however, no such precaution was taken and ink leaked into the subarachnoid space over the vertex of the left cerebral hemisphere. Even from the shortest time between Indian ink injection and death of the animal (5 min), ink had drained through the subarachnoid space and was observed in cervical lymph nodes. The distinct subarachnoid pathways of drainage for the tracer are seen on the surface of the rat brain illustrated in Fig. 4. Indian ink from the vertex of the hemisphere has passed in broad channels beside branches of the middle cerebral artery (Fig. 4a) towards the base of the brain (Fig. 4b). As this animal was injected 4 weeks previously, most of the ink is in macrophages and is punctate in its appearance. Some pooling of ink has occurred near the circle of Willis, just lateral to the optic chiasm but a narrow, distinct line of Indian ink extends forwards from the origin of the middle cerebral artery along the undersurface of the frontal lobe to the olfactory bulb. This demonstrates a highly selective, directional flow of Indian ink from the base of the brain forwards, within the subarachnoid space, to the olfactory bulb and the region of the cribriform plate.

By light microscopy, ink associated with major vessels such as the middle cerebral artery, as seen in Fig. 4, lay within the subarachnoid space (Fig. 5). Ink can be seen within macrophages associated with the pia mater and with arachnoid trabeculae crossing the subarachnoid space. Particles of ink are also seen within arachnoid cells. The ink is not intimately associated with the vessel wall nor is it in the true perivascular space.

Examination of decalcified sections of the cribriform plates of the ethmoid bone from animals in which ink had spread through the subarachnoid space revealed Indian ink in submucosal lymphatics. Ink had also spread to deep and superficial cervical lymph nodes but no ink was detected in sections of para-aortic or inguinal lymph nodes or lung in these animals.

Discussion

The present study has shown that when the particulate tracer, Indian ink, is injected into the rat brain, it spreads diffusely through white matter but is selectively distributed along perivascular spaces in grey matter. Indian ink particles are cleared from the perivascular spaces by perivascular cells which are initially closely associated with the vessel wall. Subsequently, particle-laden macrophages, possibly derived from the perivascular cells, lie immobile within perivascular spaces. Little movement of such perivascular macrophages is seen in the subsequent 2 years.

Our experiments have also shown that Indian ink injected into the cerebrospinal fluid over the vertex of the rat brain drains rapidly along selective and directional pathways within the subarachnoid space following major vessels to the base of the brain and then passing forward, again in selected subarachnoid pathways, to the region of the cribriform plate. Thence, the tracer drains into nasal lymphatics and cervical lymph nodes.

As with the Indian ink in the present study, extravasated protein in oedema fluid [16] and proteins injected into the white matter of the rat brain [21] spread diffusely through the white matter and appear to drain into the ventricular cerebrospinal fluid. This pathway of fluid drainage appears to be inhibited in acute hydrocephalus, resulting in the periventricular oedema of white matter recognised in experimental animals [4, 25, 26] and in the human brain [27]. Such oedema selectively involves the white matter and is associated with tissue damage; the grey matter in experimental and human hydrocephalus is less severely affected and this may be a reflection of the different pathways of interstitial fluid drainage seen in grey and white matter.

Selective drainage of interstitial fluid and tracers along perivascular spaces in the grey matter has been reported in previous, more physiological, experiments. When 0.5 µl Dextran Blue 2000 in a balanced salt solution was infused slowly into the caudate nucleus of the rat brain, dye spread extensively during the next 24 h and was associated with the walls of blood vessels [6]. Some selective accumulation of protein from oedema fluid was also observed in perivascular spaces by Kalimo et al. [16] and by Ohata et al. [21]. These observations suggest that the perivascular spaces outlined by the injection of particulate carbon in the present study are, in fact, routes of fluid drainage under physiological and pathological conditions.

Perivascular cells play a major role in clearing particulate matter from the perivascular spaces. By 7 days after injection, Indian ink in the perivascular spaces had been ingested by perivascular cells. By 4–6 weeks, perivascular spaces were virtually clear of Indian ink which by then resided within macrophages and perivascular cells. A few particles could be seen in leptomeningeal cells. Under physiological conditions, perivascular cells are ideally placed for clearing particulate matter and protein from fluid draining along perivascular spaces. Perivascular cells in the rat have been defined by their immunophenotype as a population that is separate from microglia and from haematogenous monocytes and macrophages [11, 12, 23]. In the present study, the perivascular cells initially remained attached to the vessel walls but subsequently became perivascular macrophages lying free within the perivascular spaces. The uptake of Indian ink from perivascular spaces in this way has previously been recorded by McKeever and Balentine [19] but they had proposed that the macrophages had migrated to the perivascular spaces from brain tissue. In that study, the authors omitted to observe the very early stages of distribution of Indian ink and thus did not recognise the way in which the tracer was distributed through the perivascular spaces at the time of injection.

In addition to uptake of Indian ink particles by perivascular cells, carbon particles were also observed within cells which resemble perivascular meningeal cells [29]. Similar uptake of particles by meningeal cells was seen in the subarachnoid space and reflects the known capacity of meningeal and meningioma cells for pinocytosis and possibly for phagocytosis [10].

The significance of the perivascular spaces in local immunological reactions has been emphasized in relation to experimental allergic encephalomyelitis [5] and in a number of human neuropathological lesions [9], but the significance may be even greater than these authors propose and may well be the main pathway by which soluble antigens or antigens within macrophages could pass to regional lymph nodes [13]. The present model of Indian ink injections into the perivascular spaces has not proved to be a successful model for observing the traffic of histiocytic cells along perivascular spaces. Even after 2 years, it appears that there has been very little movement of perivascular macrophages along the perivascular spaces; however, this may be due to the nature of the material ingested.

Once Indian ink is released into the subarachnoid space it travels rapidly along well-defined paravascular pathways in the subarachnoid space to the base of the brain and thence in a well-defined channel in the subarachnoid space to the region of the cribriform plate. Such subarachnoid channels are probably directed by the presence of leptomeningeal trabeculae that traverse the subarachnoid space both in the rat [17] and in man [1, 14]. Similar compartmentalisation of the subarachnoid space is also seen in the human spinal cord [20]. The discrete nature of these pathways suggests that there may be counter currents within the subarachnoid space, possibly with fluid flowing in different directions towards different drainage sites and that these channels are firmly separated by arachnoid trabeculae.

Although the spread of tracer along perivascular spaces in the brain is well demonstrated by Indian ink injections, and passage of the tracer through the subarachnoid space was well defined, the connection between the perivascular spaces and the subarachnoid space has not been clearly demonstrated in the present study. However, the results of previous studies do suggest that interstitial fluid drainage along perivascular spaces in the grey matter passes into the subarachnoid space. Ohata et al. [21] observed movement of protein, and thus oedema fluid, towards the cortical surfaces and the cerebrospinal fluid. More specifically, results of experiments reported by Szentistvanyi et al. [24] and by Ichimura et al. [15] suggested that tracers drain from the brain along perivascular sleeves of the vessels in the brain and in the subarachnoid space. Anatomical connections between the perivascular spaces around vessels in the brain and those in the subarachnoid space have been demonstrated in man by Zhang et al. [29]. Gaps between the meningeal cells coating the perivascular spaces of vessels in the subarachnoid space suggest that fluid leaks into the subarachnoid space rather than remaining in true perivascular spaces [29].

In summary, it would seem from the results of the present and previous studies that, in the rat, interstitial fluid from the white matter drains into the ventricles. Interstitial fluid from the grey matter, on the other hand, drains along perivascular spaces, leaks into the paravascular compartments in the subarachnoid space and passes into well-defined channels to the circle of Willis at the base of the brain and then to the region of the cribriform plate of the ethmoid in the rat. The rapidity with which tracers pass from the subarachnoid spaces to cervical lymph nodes [28] and the importance of this drainage pathway in some species [2] suggests that there are open channels between the subarachnoid space and the nasal lymphatics for the bulk flow of cerebrospinal fluid. Previously, only direct pathways of drainage by sheaths of olfactory nerves had been identified [8], but recent investigations using Indian ink as a tracer have demonstrated substantial and direct connections between the subarachnoid space and nasal lymphatics through channels within the arachnoid itself (Pantazis

and Weller, in preparation). Such pathways for fluid drainage would be compatible with the observed rapidity of the bulk flow mechanism. It is possible that such pathways also serve for drainage of lymphocytes and macrophages from the brain to regional lymph nodes.

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