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Time-Dependency of the Lymphatic Efflux of intracerebrally Applied Corpuscular Tracers

Light and Electron Microscopic Investigations

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Summary

Morphologic methods were used to investigate the rate at which intracerebrally injected corpuscular tracers (carbon, ferritin, and colloidal gold) appear in the cervical lymph nodes of rabbits. The survival intervals ranged from 3 minutes to 90 days. The lymph nodes were examined with light microscopy and sometimes also with electron microscopy.

Ferritin was first detected in the deep paratracheal cervical lymph nodes 6 minutes after injection with electron microscopy and 10 minutes after injection with light microscopy. All tracers appeared first extracellularly in the sinuses of the lymph nodes; within 3 hours, however, they had been almost entirely incorporated, appearing then in the sinus lining cells as well as in the lymph node macrophages. In the following days, the tracer concentrated and accumulated in the macrophages. After 12 days, all tracer-bearing macrophages had already disappeared. The amount and distribution of tracer in the sinus lining cells, however, remained virtually unchanged during the entire 3-month observation period. The importance of these observations is discussed.

Introduction

In 1895, *Key* and *Retzius* suggested that the CSF spaces are connected with lymphatic organs by means of pathways. Many recent studies have established the existance of such pathways (for survey, see *Millen* and *Woollam,* 1962; *Földi* 1972, *Kozma* et al. 1972). The authors' investigations confirmed a real efflux of intracerebrally injected blood cells (*Oehmichen,* 1978; *Oehmichen* et al. 1979).

Isolated observations tend to indicate that CSF clearance is time dependent, but specific experiments are not available. In the present study, semiquantitative morphological methods were used to examine the time-dependent appearance of different traces after intracerebral injection in rabbits.

Material and Methods

Experimental animals: "German Giant" rabbits (no inbred breeds; age, 6–12 months; body weight, 2000–2500 g) were used for all tests. The animals were fed with water and altromin ad libitum.

Operative procedure: The animals were anesthetized with ether/pentobarbital. After incising the scalp and exposing the cranium, a dental burr was used to drill a hole over the left hemisphere, 2 mm from the midline, under preservation of the dura mater. 0.05 ml tracer solution was slowly injected through the intact dura mater, avoiding extradural deposition of tracer. The burr hole was then closed with sealing wax, and the skin wound sutured under sterile conditions.

Survival intervals: After injection of tracer, the animals were allowed to survive for various intervals (3, 6, 10, 12, 20, and 60 minutes; 2, 3 8, 12, 24, 48, 72, and 96 hours; 6, 12, 30 and 90 days). Different survival intervals were selected for various tracers. Two animals were

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killed for each survival interval by intravenous injection of an overdose of pentabarbital.

Organ preparation: The following organs were isolated and preserved: brain with optic nerves and eyes, superficial and deep paratracheal and submandibular cervical lymph nodes, and inguinal lymph nodes.

Control experiments: The control animals received either an intracerebral injection of 0.05 ml physiologic saline solution or an intravenous injection of tracer in the ear vein. The survival intervals of these animals were the same as those of animals with intracerebrally applied tracer. The cervical and inguinal lymph nodes of these animals were preserved and examined.

Tracer: The following tracers were used:
1. Carbon
Drawing ink (17 Black, Pelikan-Günther Wagner, Hannover/FRG; 87.8 % water, 4.5 % shellac, 3.1 % gelatin, 1.5 % borax, 0.9 % phenol, and 6.2 % carbon; particle size, approx. 25 nm) was used. The ink (code C 11/1431, Pelikan-Günther Wagner, Hannover/FRG) employed since 1952 (*Biozzi* et al. 1952; *Benaceraf* et al., 1954) has now been taken off the market. The new ink functioned satisfactorily in similar experiments (*Joel* et al. 1978).

2. Ferritin

Ferritin was provided by a sterile, 10% aqueous solution of cadmium-free equine spleen (Serva Feinbiochemica, Heidelberg/FRG; molecular weight, 450; particle size, 11 nm). It is a satisfactory tracer for light microscopic (detectable with the Prussian blue reaction) and electron microscopic studies. For electron microscopic demonstration of ferritin, unstained ultrathin sections with low contrast of the normal cytoplasmic structure were used so that the iron core of ferritin could be easily detected. The ultrastructural uniformity of the ferritin core (approx. 6 nm) allows the differentiation of sporadically scattered nonspecific precipitates or beam contamination (*Sturgeon* and *Shoden*, 1969; *Fedorko* et al., 1973).

3. Colloidal gold

The colloidal gold solution (concentration, 20–50 mg/l; particle size, 35 nm) was produced according to the specifications described by *Umland* and *Adam* (1969). According to the literature, colloidal gold also functions satisfactorily as tracer, particularly for electron microscopy (*Faulk* and *Taylor,* 1971; *Ghadially* et al., 1977; *Bendayan,* 1980). *Horisberger* (1979) published a survey on the possibilities of applying colloidal gold as tracer. Colloidal gold is superior to ferritin because of its easy detectability with electron microscopy, even on stained ultrathin sections.

Histologic procedure: After cardial perfusion with a warm, buffered, 4% solution of paraformaldehyde, the brain and lymph nodes were isolated. One half of the lymph nodes were embedded in paraplast; the other half, after osmification, in araldite. 3- to 5-mμ-thick paraffin, semithin, and ultrathin sections were then cut. Ferritin was demonstrated in the paraplast-embedded material via the Prussian blue reaction. The semithin sections were stained with toluidin blue. Some ultrathin sections were contrasted with uranyl acetate; some were not.

Semiquantitative analysis: The amount of tracer per lymph node was determined comparatively on the section using light microscopy. Even though this method is relatively rough, it does provide an indication of the tendency. The findings were characterized as follows:

– = no tracer detectable
+ = single particles of tracer detectable extracellularly or intracellularly
++ = circumscribed accumulation of tracer in the lymph nodes, most of which is distributed intracellularly
+++ = diffuse distribution of many tracer-bearing cells on cut surface of all sections

Results

1. Control experiments

No tracer particles were observed in the inguinal lymph nodes of any of the animals after intracerebral injection or in the lymph nodes after intravenous injection. No pathologic alterations were found in the cervical lymph

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Fig. 1a

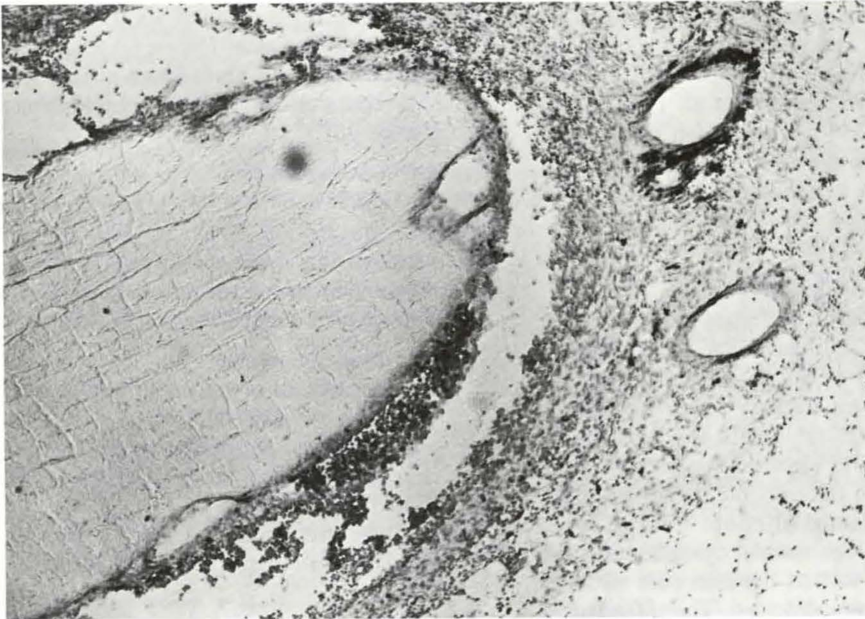

Fig. 1b

Fig. 1 Cerebral alterations after intracerebral application of ferritin. (a) Already 10 min after the tracer injection tracer-positive astrocytes are visible at the injection site (prussian blue reaction; x 1,200).
(b) Mesenchymal cells of the retrobulbar tissue ingest ferritin within 3 min after the intracerebral injection (Prussian blue reaction, nuclear red stain; x 300)

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nodes after intracerebral injection of physiologic saline solution. Ferritin-like substances, in particular, were not detected extracellularly or intracellularly.

2. Cerebral alterations

In the brain, all tracers were observed first within the CSF spaces, i.e. subarachnoid space, perivascular space, and occasionally the ventricles. Tracer was also always present at the injection site. A few tracer-bearing astrocytes appeared in the peripheral cerebral tissue 10 minutes after injection (Fig. 1a). Accumulations of macrophages were found at the injection site after 2 to 10 days. The large amount of macrophages appearing here as well as the perivascular and subarachnoidal macrophages were characterized by a massive phagocytosis of tracer.

The leptomeninges of the optic nerves were packed with tracer. Tracer particles were demonstrated in the extracerebral tissue, and therefore in the retrobulbar tissue (Fig. 1b) with light microscopy 3 minutes after injection. Single tracer-bearing mesenchymal cells appeared within the nerves several hours later.

3. Lymphatic efflux

3.1 Carbon

Intracerebrally injected carbon was tolerated differently by the animals. Since 3 animals died with signs of seizures, this tracer was studied in relatively few animals. The shortest time interval examined was 60 minutes. Tracer was detected in the deep paratracheal cervical lymph nodes 60 minutes after injection. The maximum amount of tracer was observed in these lymph nodes after 8 hours; it was still detectable 6 days later. Thereafter, the quantity of tracer particles in the section declined gradually. Tracer was still detectable in the lymph nodes of an animal which survived for 30 days, the longest survival interval studied after carbon injection.

Some particles were found in the marginal and medullary sinuses one hour after injection;

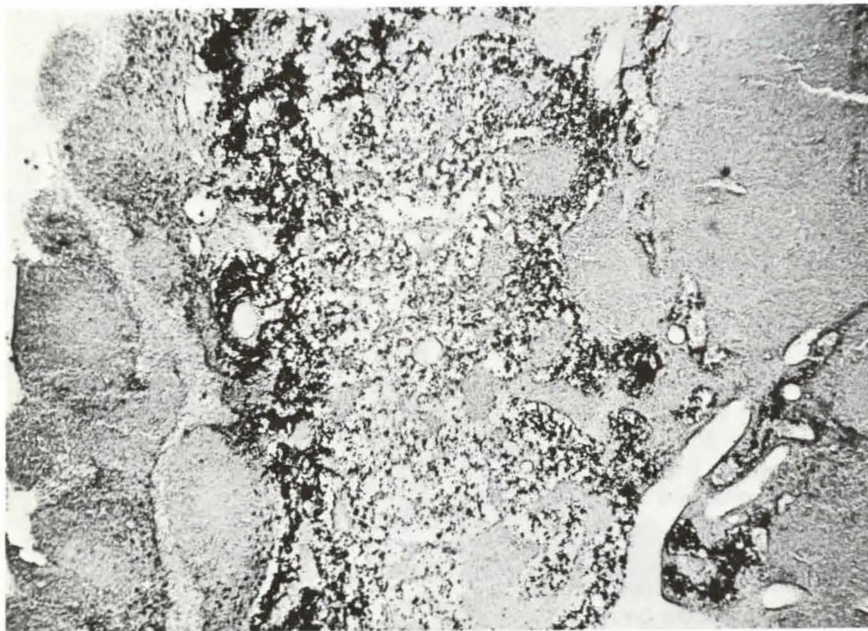

Fig. 2a

Fig. 2 Appearance of carbon in the cervical lymph nodes and ingestion of this tracer by local mesenchymal cells. (a) Demonstration of the carbon distribution in the lymph nodes 3 hours after the intracerebral injection (Hematoxylin and Eosin stain; x 40). (b) Within the processes of sinus lining cells single carbon particles are visible 30 days after tracer application (uranylacetat stain; x 6,000)

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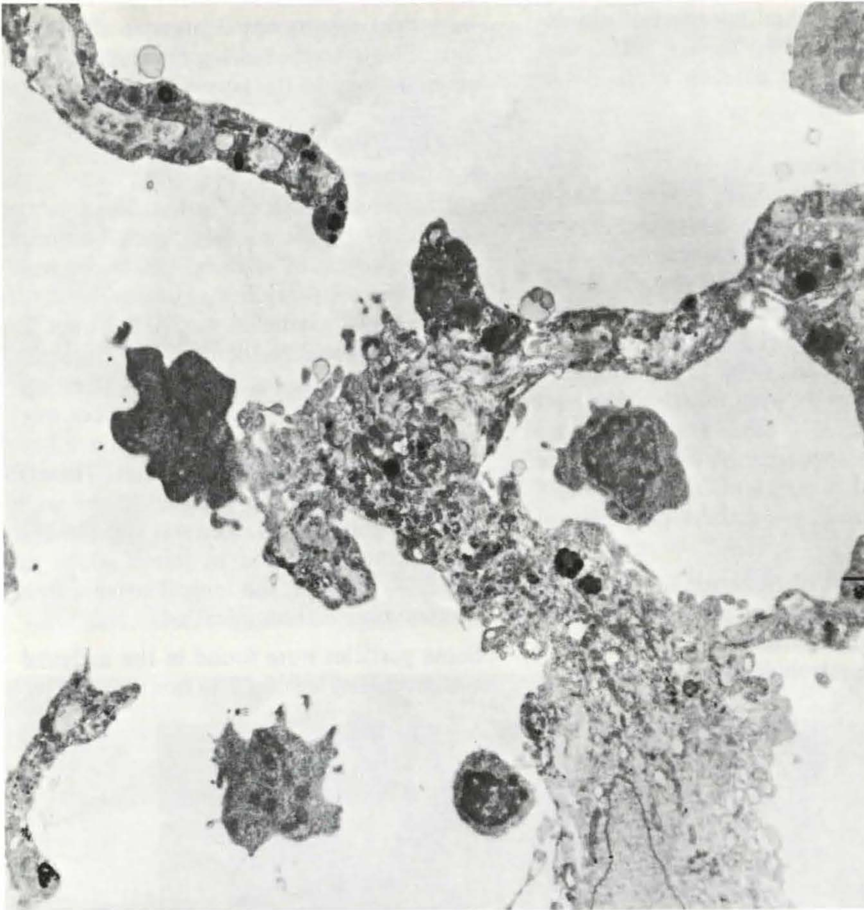

Fig. 2b

some had already been incorporated by the cells. Extracellular tracer was no longer observable with light microscopy 3 hours after injection. Tracer was incorporated particularly by sinus lining cells and macrophages within the sinus (Fig. 2a). Diffuse distribution of tracer was observed initially in both cell types using light microscopic criteria. Tracer particles accumulated in the macrophages 12 hours after injection; the diffusely distributed tracer in the sinus lining cells, however, remained unchanged. While tracer-bearing macrophages disappeared almost entirely from the lymph nodes within 12 days, incorporated carbon particles persisted in the sinus lining cells of the animals allowed to survive for 30 days (Fig. 2b).

A comparison of cervical lymph nodes from different locations showed that tracer was regularly contained primarily in the bilateral deep paratracheal cervical lymph nodes. The other lymph nodes contained less tracer and tracer was not regularly detectable in them.

3.2. Ferritin

Light microscopic examination showed homogeneous distribution of bluestained ferritin particles within the sinus at the marginal and medullary zones (Prussian blue reaction) 10 minutes after intracerebral injection. At this time, preponderantly extracellular ferritin particles were demonstrable in the marginal and medullary sinuses. Marked phagocytosis was already detectable after 20 minutes (Fig. 3a),

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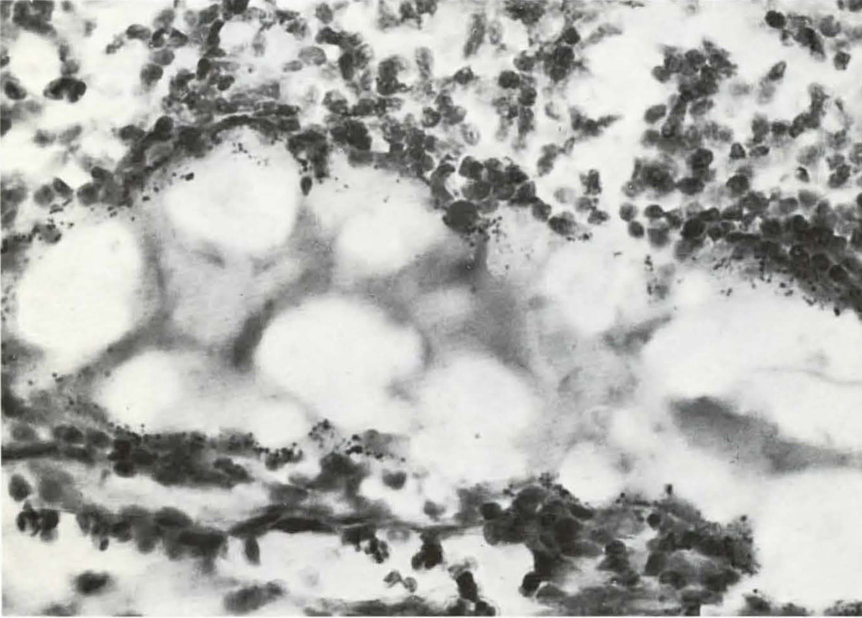

Fig. 3a

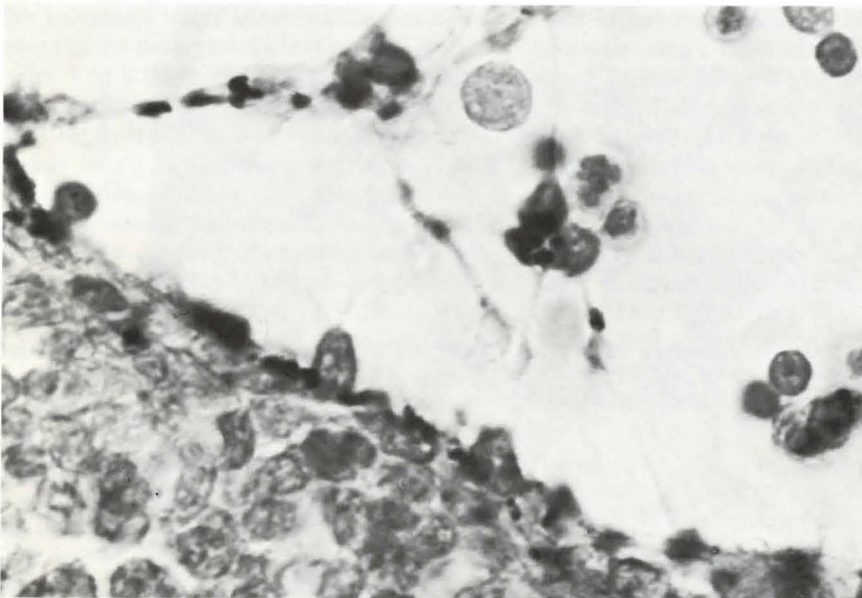

Fig. 3b

Fig. 3 Light microscopic appearance of ferritin in the cervical lymph nodes and ingestion of this tracer by local mesenchymal cells. (a) 20 min after the tracer application in the lymph node sinus ferritin particles are seen, partly on the surface of, partly still ingested by sinus lining cells (Prussian blue reaction, Hematoxylin stain; x 500). The tracer is ingested by sinus lining cells (b) and by macrophages (c) as well (Prussian blue reaction, nuclear red stain; x 1,200). (d) Tracer distribution within the cervical lymph node (only sinus lining cells) 6 days after the intracerebral injection (Prussian blue reaction, Hematoxylin stain; x 300)

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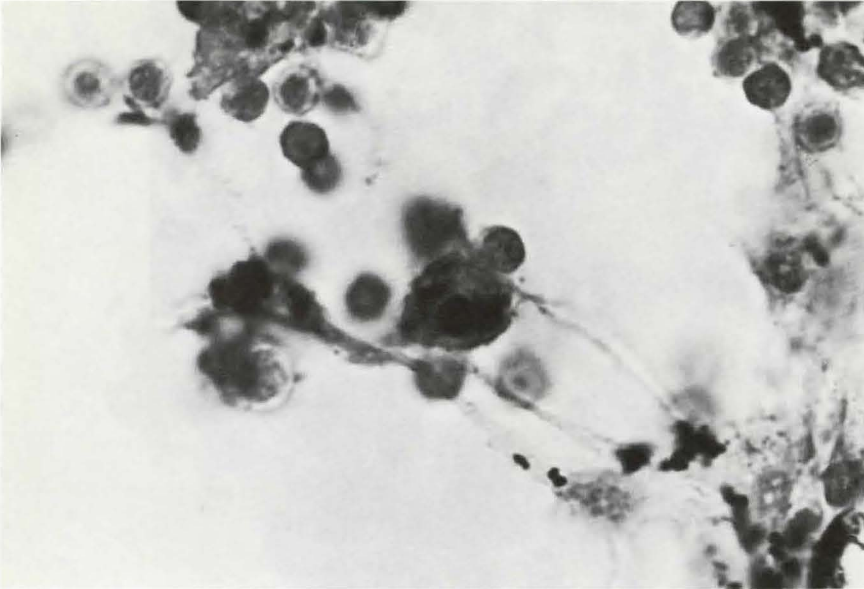

Fig. 3c

Fig. 3d

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Table 1 Semiquantitative, light microscopic analysis of the ferritin concentration on histologic sections of lymph nodes from animals surviving intracerebral injection of ferritin for different time intervals

Survival interval after intracerebral injection	Lymph nodes		
	submandibular	superficial paratracheal	deep paratracheal
10 min	–	–	+
20 min	–	–	+
1 hr	–	–	++
2 hr	–	–	++/+++
3 hr	–	–	++/+++
8 hr	+	–	+++
12 hr	+	–/+	+++
24 hr	+	+	+++
48 hr	+	–/+	+++
72 hr	+	–	++/+++
96 hr	+	+	+++
6 days	+	–	++/+++
12 days	–	–	+/++

the phagocytizing particles of tracer was seen in the cytoplasm of the sinus lining cells and macrophages (Figs. 3 b, c).
The maximum tracer accumulation was observed in the deep paratracheal cervical lymph nodes 3 to 8 hours after injection. Thereafter the quantity of tracer remained virtually constant for 6 days (Fig. 3d) and then decreased slightly over a survival interval of 6 to 12 days (comp. Table 1). While the tracer particles in the sinus lining cells were also detectable at the end of a 3-month observation period, the tracer-bearing macrophages had disappeared completely within 12 days.

Ferritin particles were clearly visible for the first time with electron microscopy 6 minutes after injection (Fig. 4). Some particles were located on the surface of the sinus-linic cells; some, however, were loosely incorporated in the endoplasmic reticulum and already forming the first siderosomes. Siderosome formation increased until, after 40 minutes, the siderosomes were tightly packed with ferritin particles (Figs. 5, 6). Extremely fine particles were also still observable in the pinoctotic vesicles and adhering to the cell surface.

3.3 Colloidal gold

Since ferritin was not detectable with electron microscopy until 6 minutes after injection, the next logical step was to study shorter survival intervals using a more easily differentiable exogenous tracer which is not normally found in the body. Colloidal gold therefore was injected intracerebrally. Two animals were examined for each survival interval (3, 6, 60 minutes). Although extracellular or intracellular gold particles could not be definitely detected 3 minutes after injection, occasional extracellular and intracellular gold particles were observed after 6 minutes (Fig. 7). A marked accumulation of tracer, however, was found in the lysosomes of sinus-lining cells and single macrophages one hour after injection.

Discussion

Our results indicate that tracer can be detected in the cervical lymph nodes shortly (i.e., within 6 minutes) after intracerebral injection. Initially, the amount of detectable tracer increases as the survival interval lengthens, reach-

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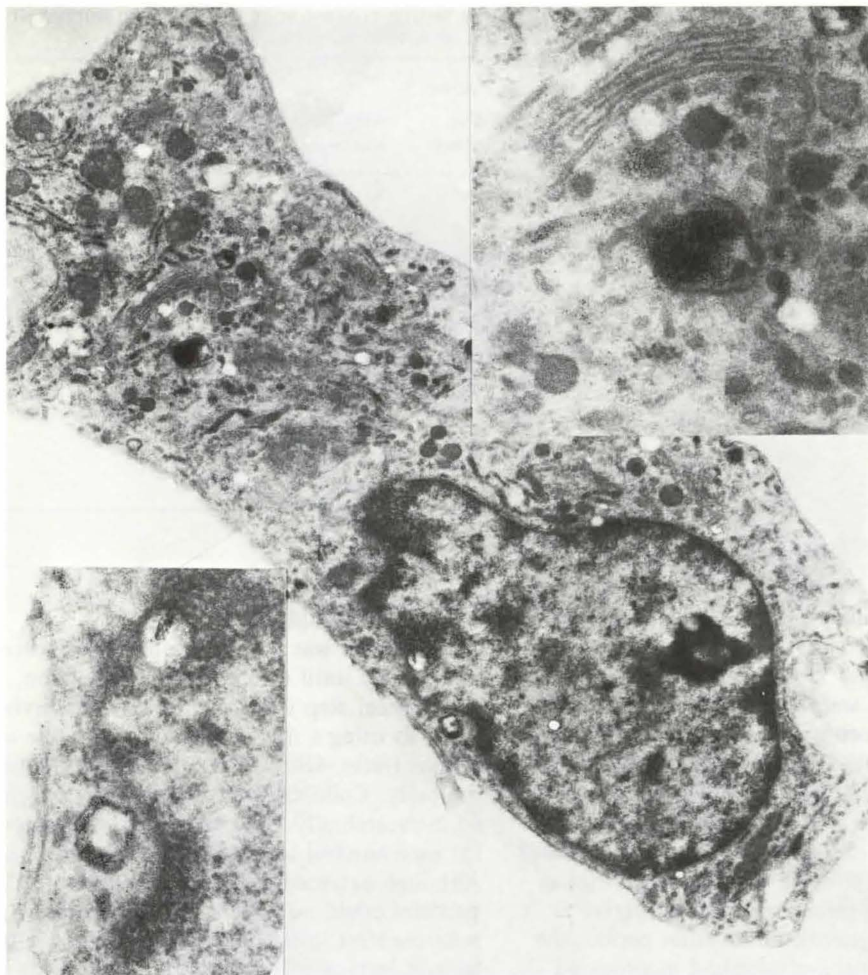

Fig. 4 Electron microscopic appearance of ingested ferritin by lymph node phagocytes 6 min after the intracerebral application: Ferritin particles are visible loosely incorporated in the endoplasmic reticulum and already forming the first siderosomes (uranylacetate stain; x 27,000 and x 70,000)

ing its maximum after 8 hours to 6 days. Thereafter, the amount of tracer gradually decreases. On the basis of these findings, it can be cloncluded that

1. Pathways are present which directly connect the CSF spaces with the lymphatic system.
2. A dependent relation apparently exists between the tracer efflux and the time interval. Tracer molecules were detectable shortly after injection. Initially, the quantity reflected a time-dependent increase and then declined.
3. Even though demonstration of tracer shortly after injection suggests that the efflux may be due to injection pressure, the kinetics indicate that a connecting pathway still exists after this time, the

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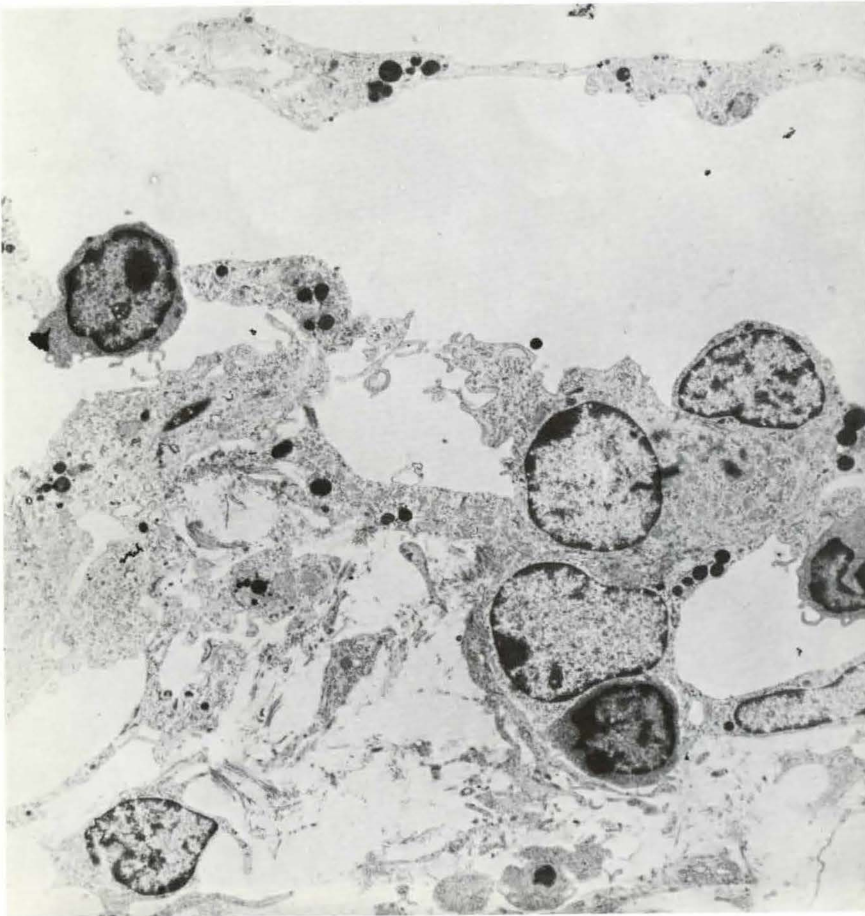

Fig. 5 40 min after intracerebral injection of ferritin typical siderosomes are visible within most of the sinus lining cells (uranylacetate stain; x 6,000)

use of which is almost totally independent of iatrogenic pressure, and in the end phase, obviously also independent of intracerebral pressure due to perifocal edema.

Our investigation therefore did not actually provide reliable answers to questions concerning the specific physiologic relations and the presence of a comparable efflux under normal conditions. Supporting studies by other authors are available (*Kleinschmidt* and *Vick,* 1976; *Bradbury* et al., 1981), but they indicate a dependency on the size of the tracer molecules (comp. *James* et al., 1976).

In each case, sinus lining cells and sinus macrophages in the lymph nodes incorporated corpuscular tracer. This observation contradicts our observations after intracerebral injection of red blood cells (*Oehmichen* et al., 1982). After intercerebral injection, autologous erythrocytes were phagocytized almost exclusively by the lymph node macrophages, but almost never by sinus lining cells. Since macrophages bearing and degrading red blood cells disappeared from the lymph nodes within 12 days, no indications of hemorrhage were detectable in the lymph nodes after this time. Tracer-bearing macrophages were also no longer

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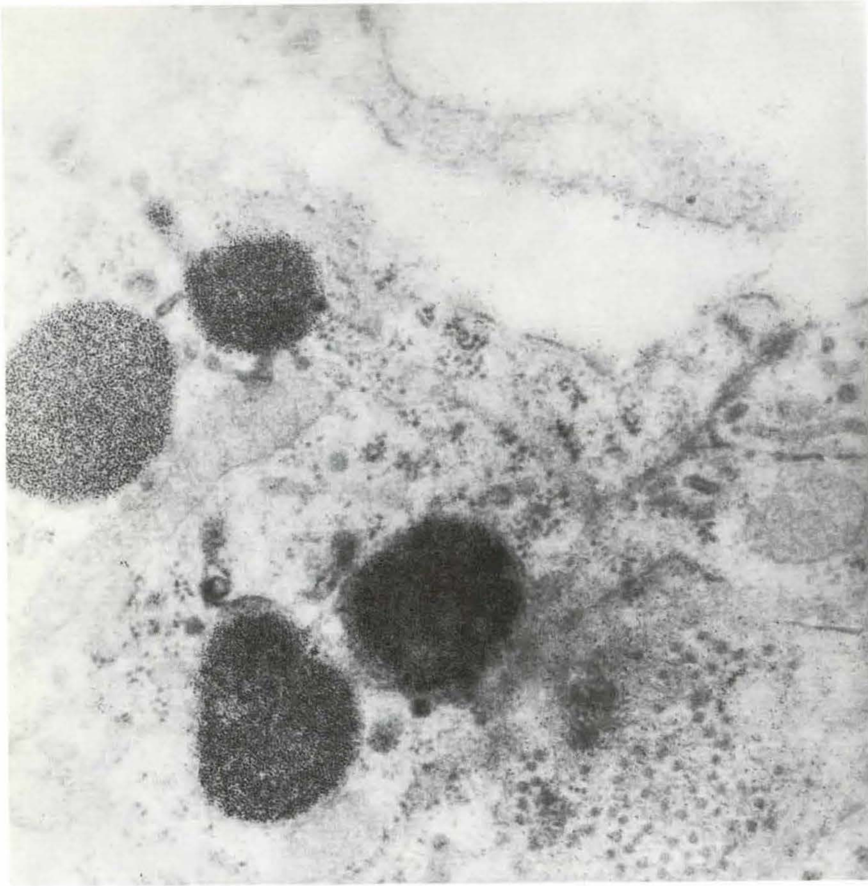

Fig. 6 Tightly packed ferritin particles are visible within the siderosomes 40 min after the intracerebral injection (uranylacetate stain; x 70,000)

present in the lymph nodes 12 days after intracerebral injectionof corpuscular tracers. Tracer incorporated by the sinus lining cells, however, was still detectable for several months.

This finding suggests that tracer-bearing macrophages disappear, but local sinus lining cells have no possibility of moving no degradating the tracer during our observation period.

Concerning the fate of the tracer, it is very possible that macrophages with incorporated tracer leave the sinus, but that sinus lining cells together with tracer persist in the lymph nodes. *Nopajaronnsrin* and *Simon* (1971) observed no tracer-bearing macrophages nor free tracer that had left the efferent part of the lymph node. These authors therefore assumed that phagocytes are destroyed in the lymph nodes and that the freed tracer is dissolved.

This observation contradicts findings reported by *Bertheussen* and coworkers (1978), who found carbon in alveolar macrophages of the lungs after intracerebral injection.

Comparison of this observation with similar experiments on extracerebral tissue indicates that, shortly after injection (subcutaneous: *Monie* and *Everett,* 1974; intraperitoneal: *Szabo* 1976, *Roser* 1970), a high concentration of tracer is found in the regional lymph

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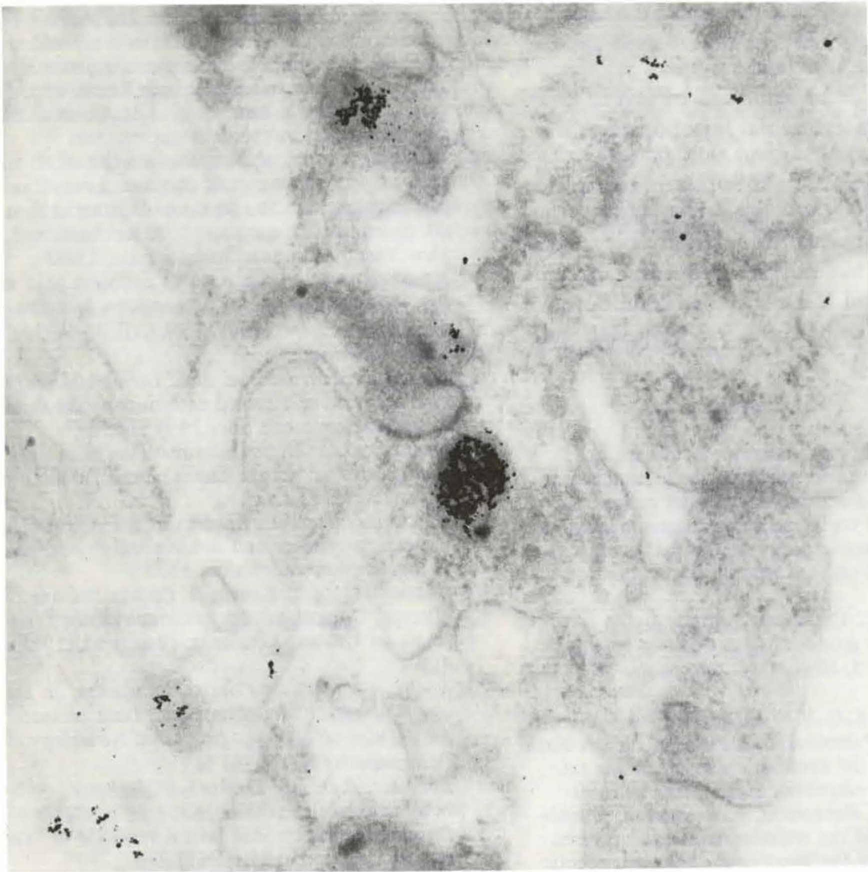

Fig. 7 Intracellular gold particles are seen 6 min after their intracerebral application within the lymph nodes phagocytes (without stain; x 70,000)

nodes, initially in the marginal sinus and later in the medullary sinus (comp. *Hoefsmit,* 1975, *Hoefsmit* et al., 1980). Our investigations support the same course for the intracerebrally applied tracers described after the intracerebral injection of autologous red blood cells (*Oehmchen* et al., 1978; 1979). The new observation in the present study is the rate at which tracer also appears in the lymph nodes after intracerebral injection.

From this point of view, mediation of the immunologic process acquires new importance. Assuming that an antigen is present within the brain, this antigen, if soluble at all or coupled to nonfixed cells, must also appear in the lymphatic system within the same time interval and produce an antigen-antibody reaction like that established in extracerebral tissue. No recent studies on this question are, in fact, available. Like *Sherwin* and coworkers (1963) after intracerebral injection of a corpuscular antigen, *Jankovic* and coworkers (1961) also detected an immune reaction in the body after intracerebral injection of heterologous red blood cells which appeared within roughly the same time interval as that observed in extracerebral tissue after application of antigen.

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In conclusion, it should be noted that both corpuscular tracer and red blood cells apparently leave the intracerebral space via the blood route. The observations of several authors after intracerebral injection of radioactively labelled red blood cells (*Kennady*, 1967, *McQueen* et al., 1974) also tend to suggest this route for corpuscular tracer. The blood route, however, is an alternative connecting pathway, but not the only one. The preferred route and the alternative conditions governing its use have not yet been clarified.

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