Cell Tissue Res (1992) 267:535–543

Cell & Tissue Research

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Cell specific DNA-labelling in the repairing blood-brain barrier of the insect *Periplaneta americana*

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Received August 13, 1991 / Accepted October 11, 1991

Summary. This study uses a recently developed technique for preserving the ultrastructure of cells in the insect CNS during immunohistochemical processing for 5-bromo-2-deoxyuridine incorporation into newly synthesised DNA. The results allow us to identify the proliferating cell classes in the regenerating blood-brain barrier. High resistance barrier cells do not label with the antibody but sheath cells clearly do. Intermediate cell types appearing during repair are identified. It is hypothesised that these cells generate matrix molecules for neural lamella repair and may represent transitional forms as invasive blood cells transdifferentiate into functional sheath cells.

Key words: Blood-brain barrier – Neural regeneration – BUdR – Glia – Cell proliferation – *Periplaneta americana* (Insecta)

Blood-brain barriers are common neural components in animals where the brain microenvironment differs significantly from the blood, as is the case in insects and mammals (Abbott and Treherne 1977). In the cockroach such a high resistance barrier (900 Ohms·cm^{-2} in the connective region: Schofield and Treherne 1984) surrounds the entire central nervous system and protects the inner compartment from marked daily fluctuations in blood potassium levels. Not surprisingly perhaps, damage to the barrier is repaired with remarkable speed and efficiency, thus maintaining the physiological integrity of the central nervous system (CNS) milieu (Smith et al. 1984, 1987).

The gross layout of the insect central nervous system, with its ganglionic chains and linking connectives, offers a unique opportunity to examine the cellular mechanisms of both neuronal and non-neuronal CNS repair (Treherne et al. 1988). Studies on the regeneration of the blood-brain barrier are made particularly simple by the accessibility of the CNS, with results implicating both endogenous glial elements and exogenous blood cells in the repair process. In several aspects both the components involved and the repair sequence share features with mammalian CNS repair (Treherne et al. 1988).

An analysis of cell proliferation during repair implies that two cell populations make up the insect blood-brain barrier and exhibit very different kinetics. Smith et al. (1990) used the incorporation of the thymidine analogue 5-bromo-2-deoxyuridine to follow mitotic activity in the penultimate abdominal connective of the cockroach at the light microscopic level. Division had been induced by a controlled chemical lesion to the connective glia. Labelling was observed throughout and beyond the lesion zone but a separate unlabelled population of neuroglia also appeared. This confirmed the results of previous studies (Treherne et al. 1987, 1988) which implied that a population of glial cells, destined to comprise a part of the repaired barrier, arose in the anterior ganglion. As yet, however, we have been unable to relate the results from these observations of cell division with either of the two types of barrier cells described by Maddrell and Treherne (1967), or the possibility that invasive blood cells become functional elements of the blood-brain barrier. The present study returns to this problem, taking advantage of our recent success in observing BUdR labelling at the ultrastructural level, while retaining enough detail for cell identification (Swales and Smith 1990).

Materials and methods

The experimental animals used in this study were male American cockroaches, *Periplaneta americana (L)*. The animals were housed at 28° C in the insect rearing facility at the Department of Zoology, Cambridge. They were fed and watered ad libitum.

Controlled lesioning and application of BUdR

Periplaneta americana was chosen as we know a considerable amount about the cellular mechanisms behind glia regeneration

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in vivo (for a review see Treherne et al. 1988). A chemical lesion can be accurately placed in the centre of the penultimate connective by use of the DNA intercalating drug ethidium bromide. This kills the glia but leaves the axons intact (Smith et al. 1984). Repair is rapid and cell proliferation can be followed by the administration of 5-Bromo-2-deoxyuridine (BUdR; Sigma), a thymidine analogue incorporated into newly synthesised DNA. BUdR can be applied as an overnight dose in the animal's drinking water ($1\ mg \cdot ml^{-1}$) and localised immunohistochemically (Smith et al. 1990). Animals were dosed with BUdR at various intervals following lesion (2, 4, 6, 8, 9, 11 and 14 days) after which they were killed and the abdominal connective dissected out in cockroach Ringer (157 mM NaCl, 3 mM KOH, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM Trehalose and 8.6 mM HEPES, pH 7.4). To minimize mechanical damage the entire length of the abdominal nerve cord was processed. In total the centre of the lesion zone was examined in 147 animals from 18 separate experiments. Further, 8 lesion zones were examined at 20 µm intervals from animals between 6 and 14 days after damage. Controls were examined for each experimental run.

Tissue processing

In the first paper on processing insect CNS tissue for ultrastructural localization of BUdR several different procedures were tested (Swales and Smith 1990). What follows below is a summary of the most effective treatments and those used in the present study.

The tissue was pre-fixed for 15–30 min in 2% paraformaldehyde plus 0.1% monomeric glutaraldehyde (Taab), in 0.1 M Sorensen's phosphate buffer with 6% sucrose at pH 7.2, or 2% paraformaldehyde plus 0.1% monomeric glutaraldehyde with 1% 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (ECD) in 0.1 M Sorensen's phosphate buffer with 6% sucrose at pH 7.2. The material was then washed in 0.1 M Sorensen's phosphate buffer with 6% sucrose at pH 7.2. Both fixations gave equally good results. While the quality of tissue preservation is poor compared to normal fixation sufficient detail of cell structure is retained to enable the identification of different cell types.

After pre-fixation, all samples were treated with 50:50 4N HCl:PBS (phosphate buffered saline) for 30 min. This was followed by washing in PBS prior to permeabilisation in 4 mM sodium deoxycholate for 10–15 min. The tissues were quenched of non-specific binding by incubation in 10% goat serum in PBS plus 0.05% saponin with $1\ mg \cdot ml^{-1}$ BSA for 1 h.

Whole prefixed, permeabilised and DNA denatured connectives were incubated intact in the primary antibody at a dilution of 1:200 in PBS with 1% goat serum, 0.05% saponin and $1\ mg \cdot ml^{-1}$ bovine serum albumin (BSA). Incubation was overnight at 4° C with continuous agitation. This was followed by washing in several changes of PBS over 2 h.

Post-fixation was with 2.5% glutaraldehyde in $0.1\ mol \cdot l^{-1}$ phosphate buffer plus 6% sucrose for 1 h. Tissue was then washed in PBS plus 6% sucrose. A further fixation with 0.5% osmium tetroxide in Sorensen's buffer plus sucrose for 30 min followed. After a brief wash in distilled water, the tissue was stained en bloc in 2% aqueous uranyl acetate, dehydrated through an ascending series of alcohols to propylene oxide and embedded in Araldite (Taab).

Sections, for both light and electron microscopy, were cut through the treatment zone in the centre of the penultimate connective with a Reichart-Jung Ultratome. Sections for second layer antibody labelling were cut onto celloidin coated nickel grids. These were labelled by floating face down on drops of solution. Some sections were etched with saturated sodium metaperiodate for 20 min, followed by further washing with PBS before antibody incubation. All preparations were incubated for 1 h in 10% goat serum in PBS with 0.05% saponin and $1\ mg \cdot ml^{-1}$ BSA. This was followed by IgG 5 nm and 10 nm gold anti-mouse, diluted 1:5 in PBS with saponin and BSA as stated above, for 2 h. The sections were then washed in PBS followed by water, stained with uranyl acetate and lead citrate before examination in a Philips EM420 at 80 kV. Gold particles of 5 nm were difficult to resolve when overlying condensed chromatin. Most of the results were acquired by use of the 10-nm gold second layer.

Several different controls were carried out. Labelling was performed on samples that had not been exposed to BUdR and on material exposed to BUdR but where few or no dividing cells would be expected. Other controls omitted the monoclonal BUdR antibody or were incubated with gold particles tagged to an inappropriate second layer antibody.

Chemicals were purchased from Sigma, with the exception of the anti-BUdR (Becton Dickenson) and the gold conjugate (BioCell). Unless stated differently all processing took place at a room temperature of 20° C.

Results

Normal structure and control labelling

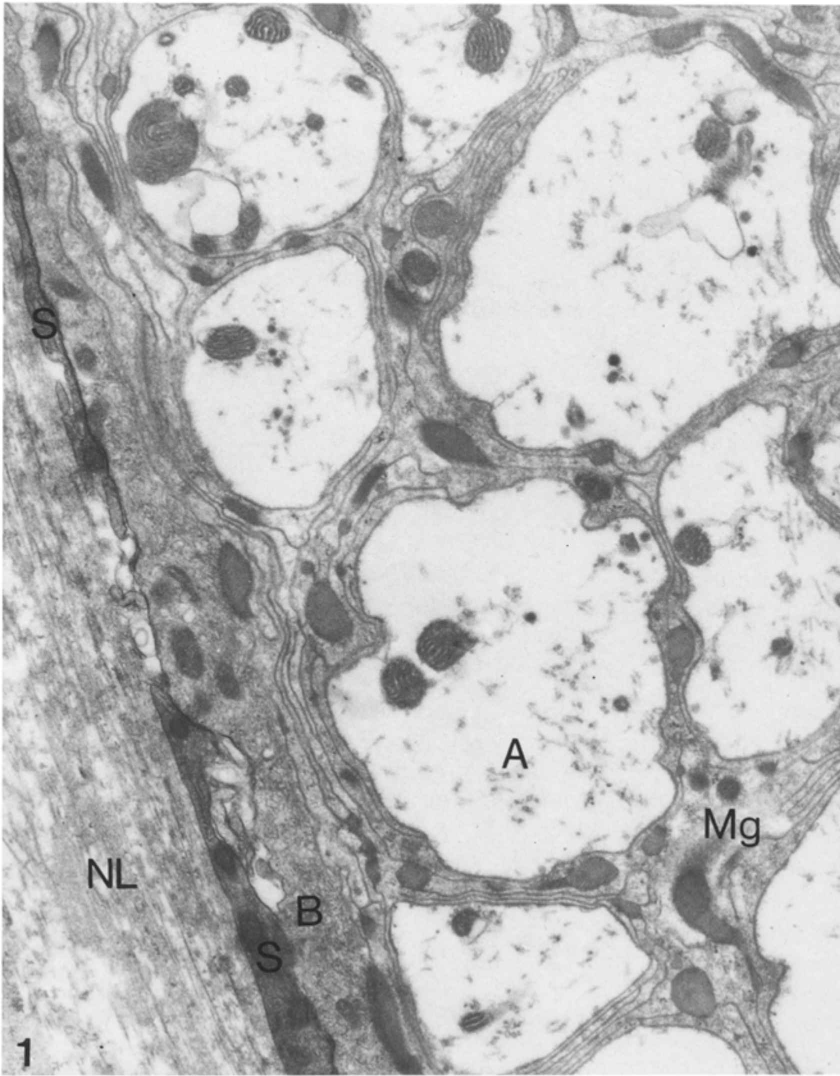
Fig. 1 illustrates a typical EM section through the connective barrier of *Periplaneta*. Two cell types are present. These cells have been referred to as the Type I and Type II perineurial cells by Maddrell and Treherne (1967) but as only Type I has been implicated in the production of the connective tissue sheath (see Scharrer 1939 and Ashhurst 1979) the term perineurial seems misleading. In this paper Type I is referred to as the sheath cell (Scharrer 1939) and Type II as the barrier cell (Smith et al. 1991). Sheath cells, which do not form a continuous layer around the connective, are characterised by numerous mitochondria, Golgi elements and endoplasmic reticulum (Fig. 2). Microtubules are relatively scarce. The barrier cells, the second cell type found in this superficial position, do form a continuous layer around the entire connective and are characterised by numerous microtubules (Fig. 2) and processes extending down into the glia surrounding the axons (not illustrated in Fig. 1: see Maddrell and Treherne 1967; Schofield et al. 1984).

After incubation with BUdR no nuclei in the control preparations were seen to be labelled by the antibody. Label was never seen when the primary layer was omitted or an incorrect second layer conjugate was used. In treated animals, when label was present, it was largely confined to the nuclei and the background was low. As polyploidy does not occur in the tissue of the connective,

Fig. 1. Normal appearance of cells beneath neural lamella of undamaged cockroach connective. Sheath cells (*S*) form discontinuous band immediately beneath neural lamella (*NL*), barrier cells (*B*) underneath these form continuous layer around axons (*A*) and mesoglia (*Mg*). ×18000

Fig. 2. Sheath cell (*S*) with characteristic cell components, mitochondria (*m*), Golgi elements (*g*) and endoplasmic reticulum (*er*), overlying microtubule-rich barrier cell (*B*). ×40500

Fig. 3. Cells at edge of connective 4 days after ethidium bromide treatment. Labelled nucleus (*N*) beneath neural lamella (*NL*) does not resemble that of either sheath or barrier cell in undamaged control. ×28500. *Inset*: Higher power of 10-nm gold particle labelling of nucleus. ×67000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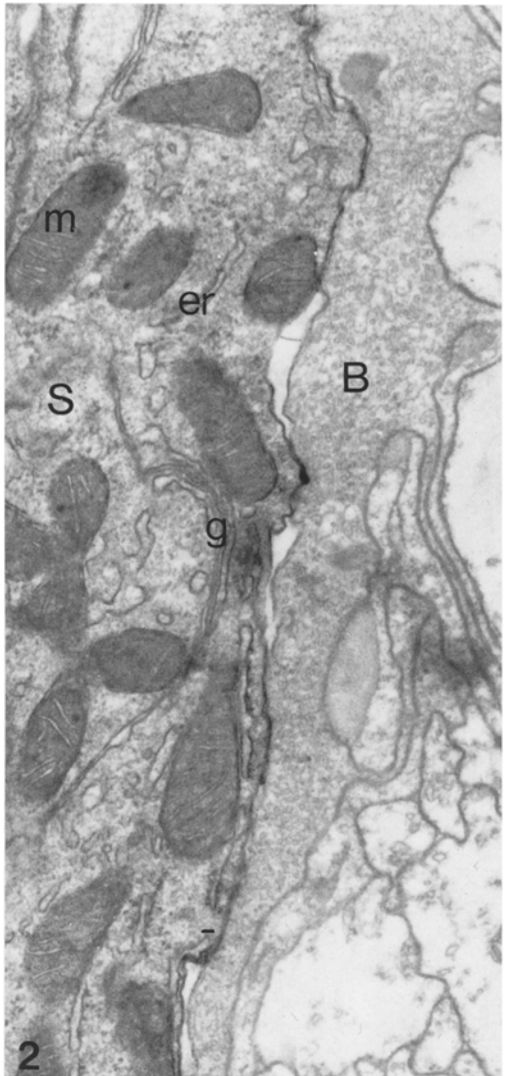
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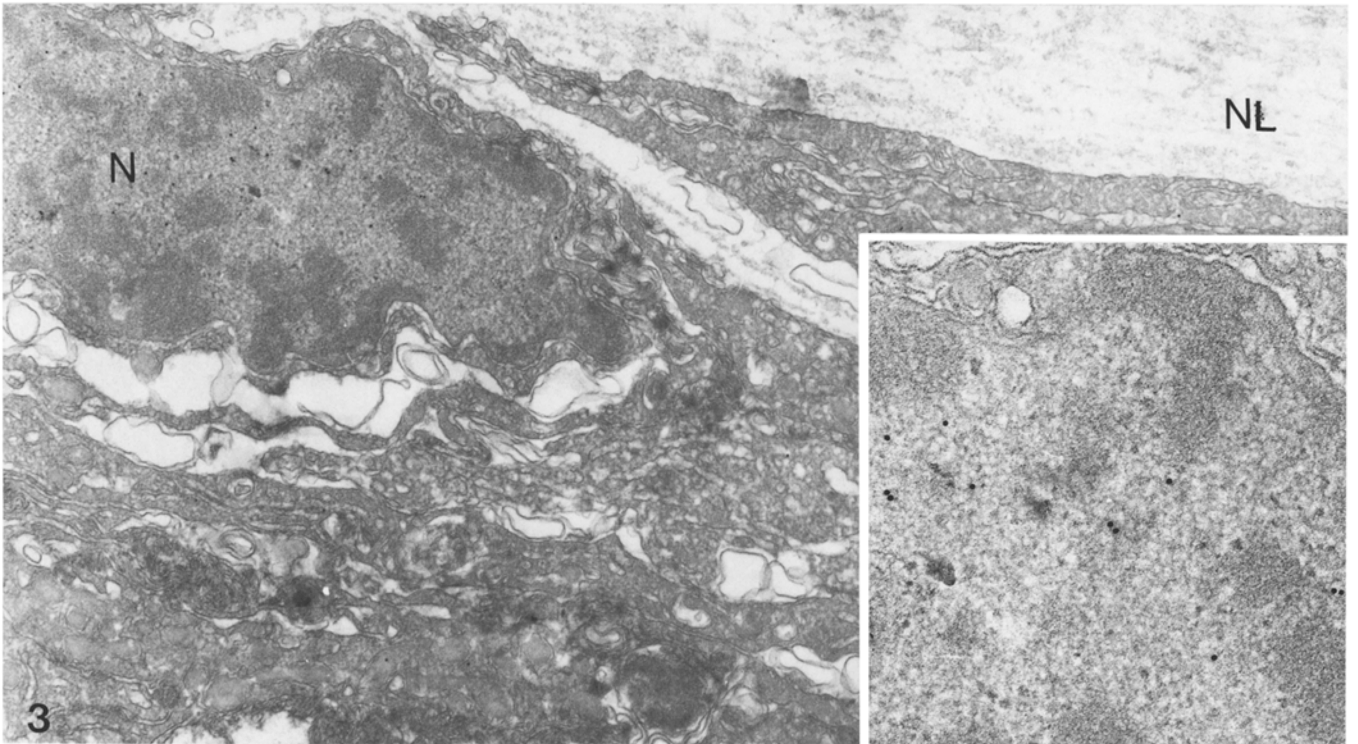
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before or after damage (Smith and Howes 1987), labelling is taken to be indicative of BUdR incorporation into the DNA molecule prior to mitosis.

Pattern of repair

Several previous publications from this laboratory have described in some detail the pattern of glial regeneration after lesioning with ethidium bromide. The time course of repair and the cellular events that occur during this process are shown in this study to follow exactly the pattern already described. What follows, therefore, is only a brief summary and more details can be obtained from various reviews (Smith et al. 1991; Treherne et al. 1987).

Application of ethidium bromide causes a rapid breakdown in the integrity of the barrier region and underlying tissues within 24 h. By two days a novel cell class appears, the granule-containing cells (gc-cells), which are never seen to label with the antibody against BUdR. Previous studies suggest that this cell class is derived from reactive haemocytes (Smith et al. 1986; Howes et al. 1987). By four days after the lesion the granule-containing cells have largely disappeared but an abnormal barrier structure remains. This structure gradually returns to a more normal configuration by 14 days.

Nuclear labelling in the barrier region

Labelling first appears on the fourth day after damage in cells situated immediately below the neural lamella (Fig. 3). Approximately 20% of the cells present are labelled by the antibody but at this stage they cannot be clearly classed as either sheath or barrier cells. Labelling is confined to the nucleus (Fig. 3, insert).

The number of labelled nuclei rise dramatically over day 6–8, with 80% of the cells in the outer area of the barrier zone being labelled. Barrier cells, clearly identifiable by their position and microtubular content (Figs. 4, 8), are now present in the lesioned zone but are never labelled by the antibody (Fig. 4 and insert). At around 6 days after lesioning another cell type, labelled and occupying the position of the sheath cells (Fig. 4) is seen. Structurally, however, it is different from the original sheath cell. The cytoplasm is electron dense with dilated rough endoplasmic reticulum and the cells may remain very attenuated. No intracellular storage vesicles are seen in these cells and there is no evidence for exocytosis. The appearance of these cells in the area of damage immediately below the neural lamella is not well synchronised. Within a single treatment zone a variety of glial cell morphologies can be seen, ranging in appearance from cells with dense cytoplasm (Fig. 5), through intermediate types with less dense cytoplasm and large amounts of rough endoplasmic reticulum (Fig. 6), to cells more closely resembling the sheath cells in untreated connectives (Fig. 7).

By 11 and 14 days post-lesion the normal structure of the blood-brain barrier has been largely re-established (Fig. 9). A discontinuous layer of sheath cells now overlies a complete sleeve of barrier cells. In both cases their cytoplasmic characteristics are those of control cells (compare Figs. 9, 10 and 12 with 1 and 2). Some labelling is still seen but the number of nuclei incorporating BUdR is greatly reduced (between 10 and 20%). Again, however, labelling is restricted to the outer cells in the repaired region of the blood-brain barrier. These now show all the cytoplasmic characteristics of sheath cells. The barrier cells are never labelled.

Some abnormalities exist in the connectives even after 14 days of repair. Areas of the blood-brain barrier are occasionally much deeper than in the controls, a feature resulting from large sheath cells lying above an apparently normal layer of barrier cells (Fig. 11). The nucleus shown in Fig. 11 (see insert) is lightly labelled with 5-nm gold conjugated antibody. A further abnormality which has not been studied in detail is the appearance of axons containing dense core vesicles above the barrier cells but within an open configuration of sheath cells (Fig. 13). Such vesicles may be indicative of neurosecretion.

Discussion

A clear result from this study is that labelling, attributed to the incorporation of BUdR during the S-phase of the cell cycle, is not distributed amongst all the cell types in the regenerating blood-brain barrier. Notably, both the granule-containing and barrier cells are unlabelled. Past work has shown us that the gc-cells are derived from invasive blood cells (Smith et al. 1986; Howes et al. 1987a) and therefore need not be recruited by division from existing populations within the brain. Where, however, are the barrier cells coming from?

Studies in this laboratory have already shown that two distinct populations of cells are involved in the regeneration of the blood-brain barrier. At the LM level only one category labelled with BUdR. The other group appeared to be recruited from a location anterior to the lesion site, perhaps in the body of the anterior gangli-

Fig. 4. Cells at edge of connective 6 days after glial damage. Barrier cell (*B*) unlabelled, electron-dense cell in normal position of sheath cell (*) labelled. ×14000. *Inset i*: Labelled nucleoplasm of electron-dense cell (*). ×55000. *Inset ii*: Nucleoplasm of barrier cell showing absence of label. ×55000

Figs. 5–7. Six days after glial damage a range of cytoplasmic morphology is shown by cells occupying area below neural lamella (*NL*) normally containing sheath cells. In **Fig. 5** cell has dense cytoplasm with aggregations of densely-staining mitochondria (*m*), and clear profiles of endoplasmic reticulum (*arrow*). ×30500. Cell in **Fig. 6** has less dense cytoplasm and contains rough endoplasmic reticulum (*arrow*). ×27000. It appears intermediate in type between cell in **Fig. 5** and cell in **Fig. 7** where cytoplasm resembles that of sheath cells in undamaged tissue. ×63500

Fig. 8. Barrier cell (*B*) from 6-day ethidium bromide-treated connective with morphology virtually indistinguishable from that of undamaged tissue. ×46000

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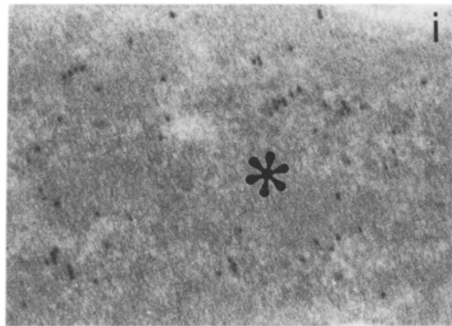
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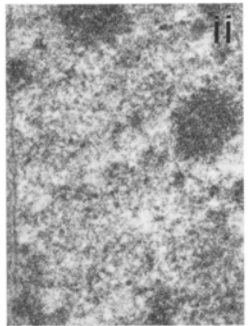
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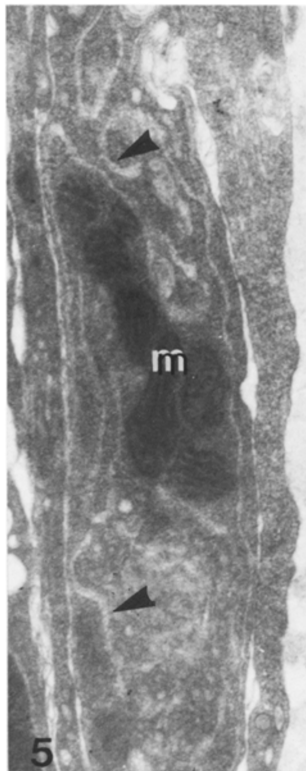
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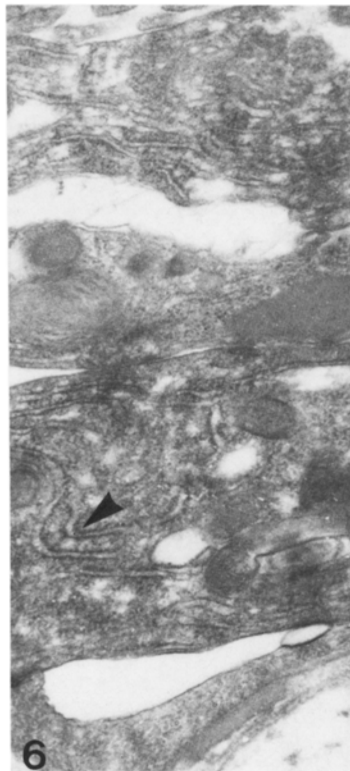
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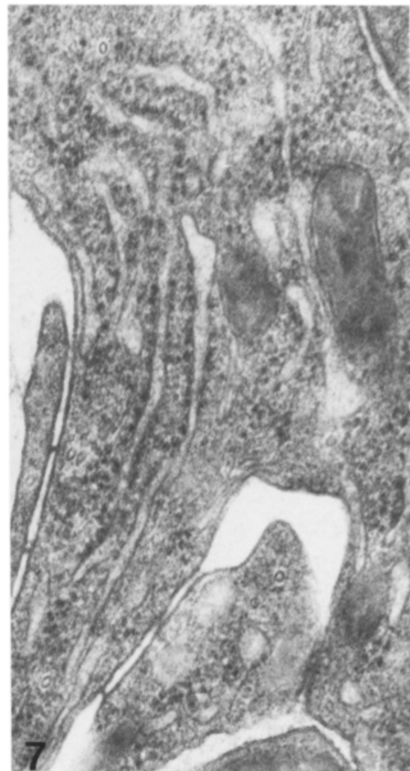
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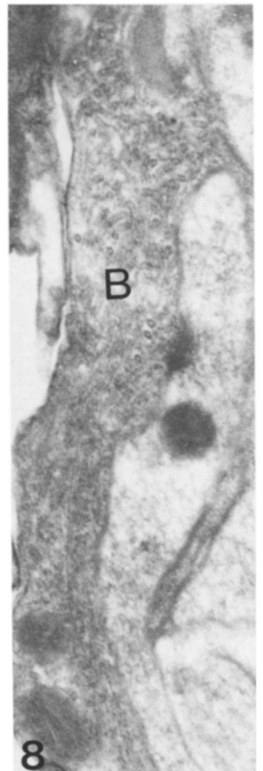
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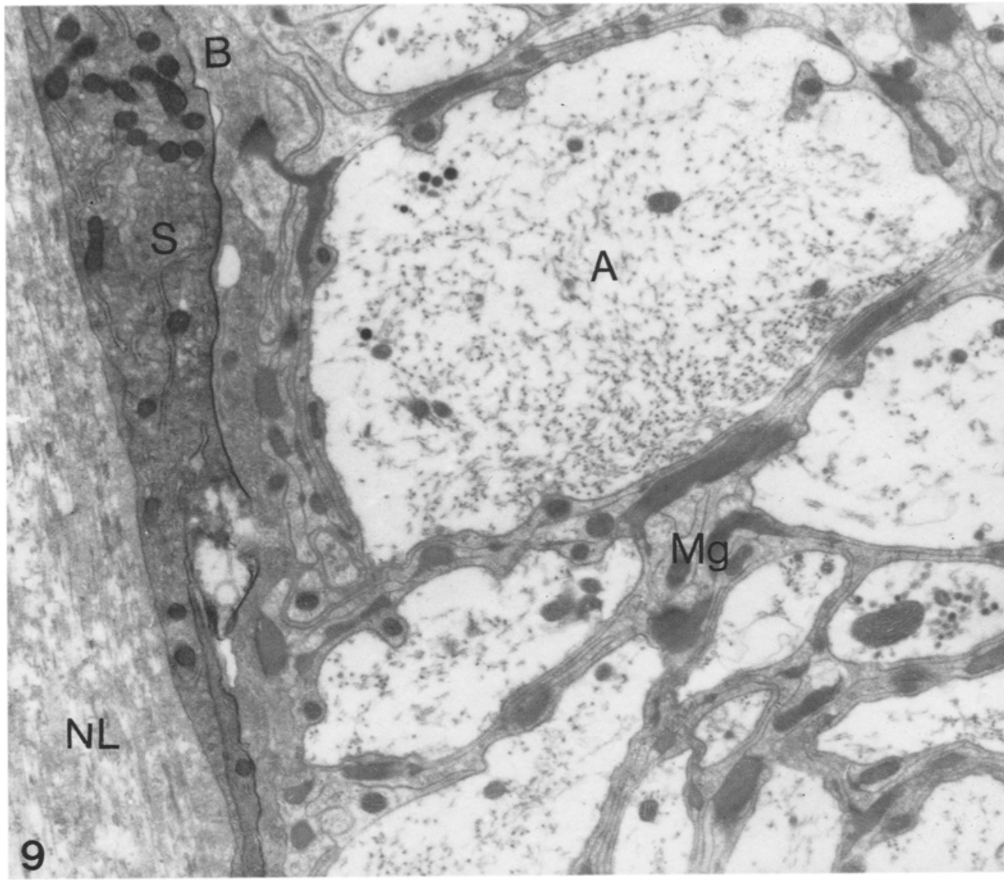
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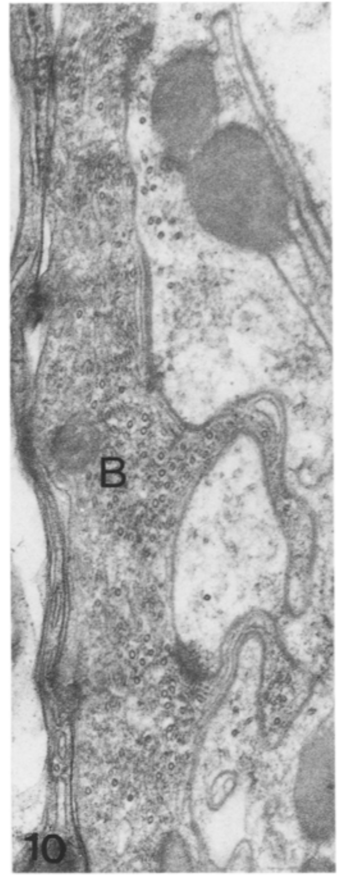
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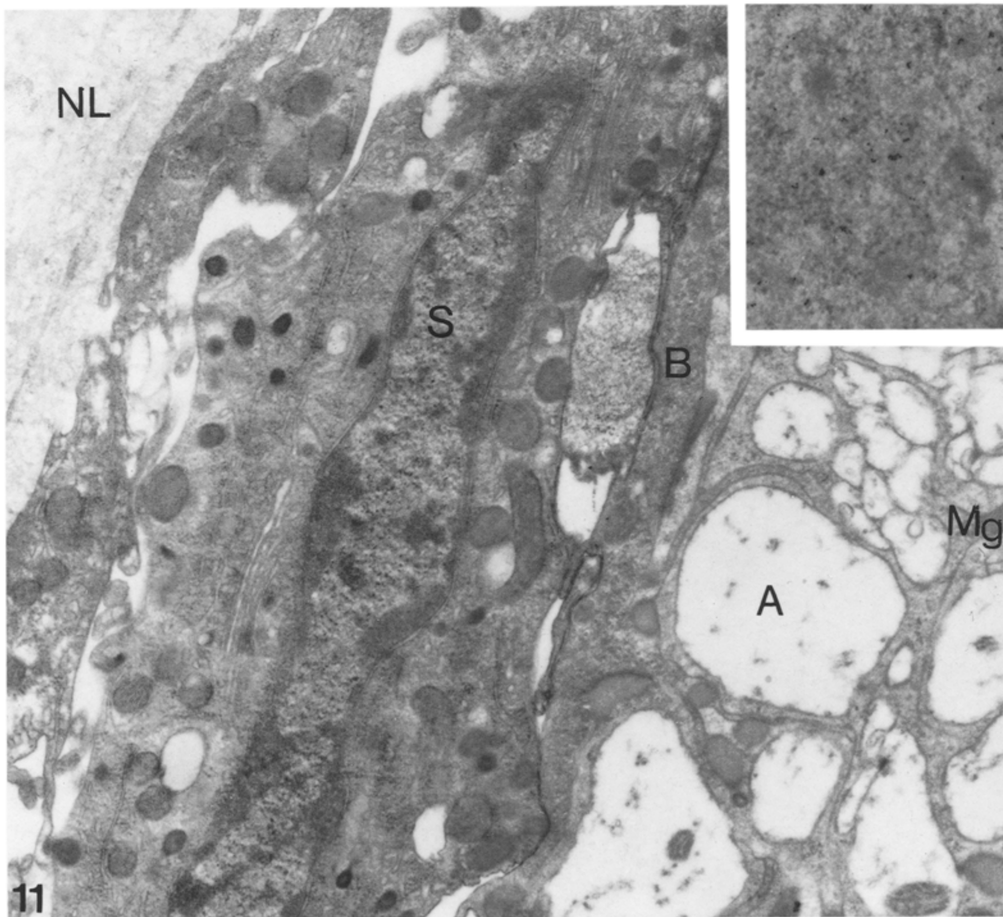
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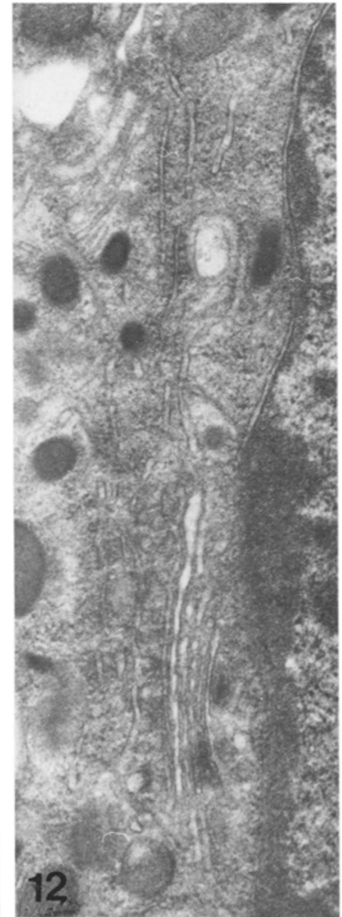
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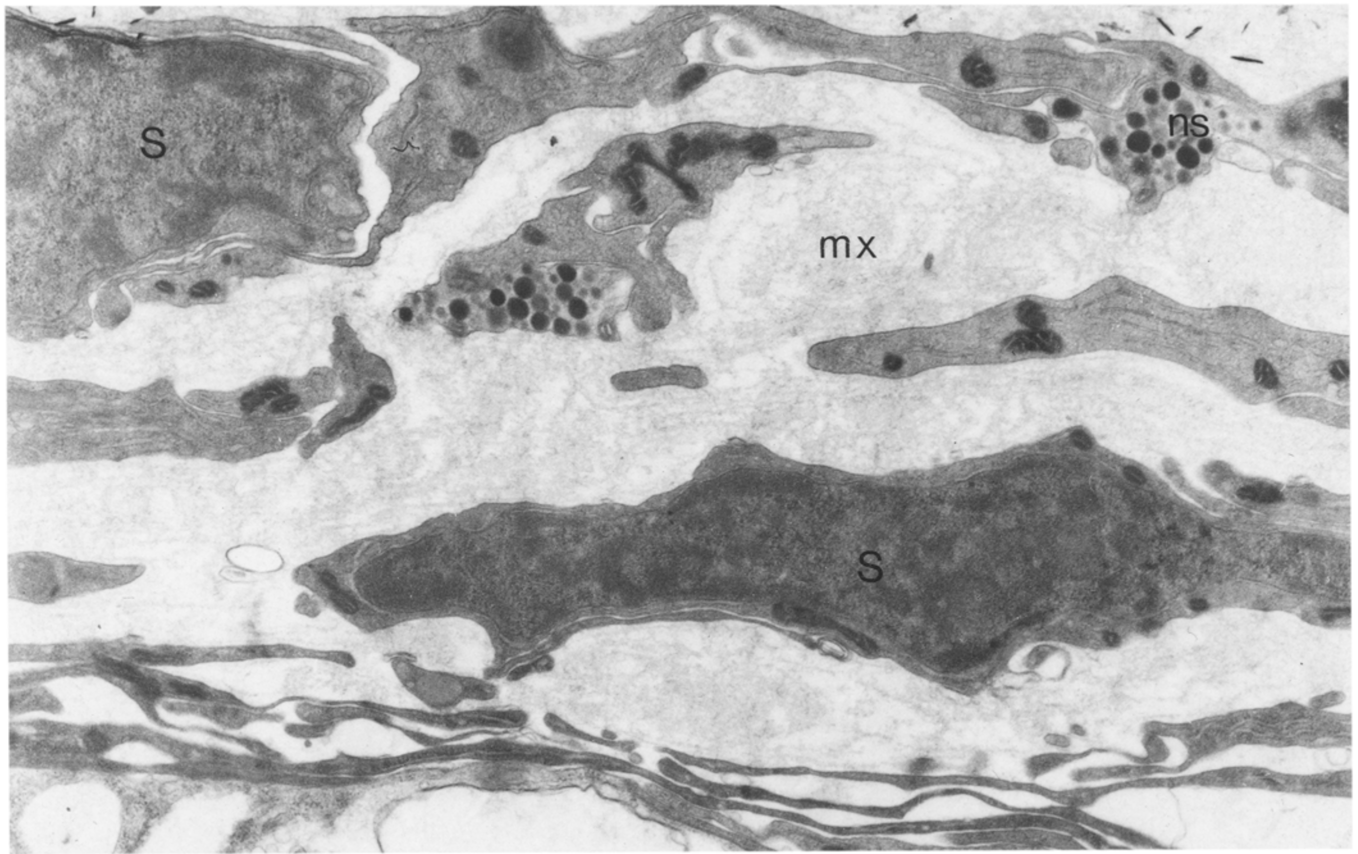

Fig. 13. Edge of 11-day treated connective with vesicle-filled neurosecretory axons (*ns*) interspersed between loosely-packed sheath cells (*S*) surrounded by extracellular matrix (*mx*). ×20500

on (Treherne et al. 1987, 1988; Smith et al. 1990). A possible developmental basis for this observation was proposed by Smith et al. (1991) who further suggested that this unlabelled group may well constitute the regenerative barrier cells. These cells appeared to fuse with the lesion zone at the same time as the blood-brain barrier began to re-establish an impermeability to potassium. These events occurred at around 6 days post-lesion – coinciding with the time that unlabelled barrier cells were first seen in the current study. This close correlation between the different studies implies that the restoration of the functional impermeability of the barrier to small cations resulted from the movement of migratory barrier cells into the lesion zone. These very specialised barrier cells did not proliferate in situ but as discussed by Smith et al. (1990) may have divided at some other location where BUdR was not available or not incorporated.

The second category of cells in the regenerating blood-brain barrier is, in many ways, more interesting. These are labelled as a result of BUdR incorporation and cell division. The time course of labelling corresponds exactly with that reported from the LM study (Smith et al. 1990) with antibody binding first appearing between 4 and 6 days post-lesion, rapidly peaking at days 6–8 but carrying on at a low level for two weeks. Data from Treherne et al. (1988) working with total cell numbers suggest that division may go on for at least one month post-lesion.

The labelled cells can be divided into three categories. The first to appear, at four days, are similar to those already described by Smith et al. (1984). They have none of the cytological characteristics of either sheath or barrier cells and, in the earlier study, are occasionally seen with electron-opaque granules. The second class of labelled cells are characteristic of the intermediate repair condition. They are present at six days post-lesion and contain dense cytoplasm and dilated rough endoplasmic reticulum. In an earlier study, where a better fixation

Fig. 9. Edge of connective 14-days after treatment with ethidium bromide. Organization of tissues in the barrier region is restored with sheath (*S*) and barrier (*B*) cells clearly present between neural lamella (*NL*) and underlying axons (*A*) and mesoglia (*Mg*). ×14000

Fig. 10. Barrier cell (**B**) from 14-day treated connective with numerous microtubules and processes extending into underlying tissues. ×39400

Fig. 11. Edge of connective from 14-day treated connective in area where sheath cell layer (*S*) is thicker than usual and barrier cells (*B*) are displaced further down into the connective. Nucleus of sheath cell is labelled. ×21500. *Inset*: Higher power of gold particles over sheath cell nucleus. ×60000

Fig. 12. Cytoplasm of sheath cell in 14-day treated connective with appearance typical of sheath cells in control tissue. ×35000

was possible, these features are more clearly seen (Treherne et al. 1988). In that study, however, they were considered atypical, being a product of interfering with blood cell function. The results presented in the current study illustrate that their involvement may be a normal part of the repair process. The last labelled category are the sheath cells clearly re-established in their proper position by 11–14 days post-lesion.

Although we know little about the role of the sheath cells in the physiology of the barrier their reappearance seems a logical outcome of the repair process. What remains more intriguing is the origin and role of the other cells involved in the earlier stages of repair. Combining the results from this and other work allows us to put forward an hypothesis.

Several studies over the past few years have established that the granule-containing cells are derived from a population of haemocytes. Further, it has been suggested that they might transform into functional sheath cells over the 14-day repair period (Smith et al. 1987; Treherne et al. 1988). This interpretation gains credence from recent developmental studies which indicate that the sheath cells in *Drosophila* are either derived from or are dependent upon mesodermal tissue (Edwards et al. 1990). Shepherd and Bate (1990) have also shown that these cells have a lineage quite different from that of the other neuroglia.

That mesodermally derived haemocytes transform into functional sheath cells of the blood-brain barrier is now a plausible hypothesis and one anticipated by Scharrer (1939), but what of the intermediate cells characterized by the presence of copious quantities of RER? Ethidium treatment causes damage to the neural lamella as illustrated by the autoradiographical studies of Smith and Howes (1984) and the EM work of Treherne et al. (1987). It is probable that the RER-rich cells appearing during repair are actively synthesising collagen and proteoglycans to replace the damaged extracellular matrices. These cells with their dilated and abundant RER bear some resemblence to the embryonic sheath cells described by Ashhurst (1965). In development such cells have been considered as the source of the CNS neural lamella (see Ashhurst 1979). The regenerating cells differ from the sheath cells, however, in having an electron-dense cytoplasm the cause of which is not known. Cells with these characteristics are not seen during glial repair in ethidium bromide-damaged and cultured connectives (Howes et al. 1987b) but a dilated and abundant RER is a characteristic shared by a class of *Periplaneta americana* haemocyte (Scharrer 1972).

Some controversy has existed over the role, if any, of haemocytes in the synthesis of the neural lamella (see Ashhurst 1979; Wigglesworth 1979; Lackie 1988). In this regard the results from the regeneration and developmental work are particularly interesting. The current hypothesis, relating results from the different studies, is that transformation of invasive blood cells to sheath cells is feasible (Smith et al. 1990). The present study suggests we might go further: invasive blood cells may be of a nondifferentiated type that, in situ, adopt the role of connective tissue synthesis, resembling both the haemocytes described by Scharrer (1972) and the developing sheath cells described by Ashhurst (1965). At this stage they are also capable of division and may have had to go through a mitosis to effect transdifferentiation. Certainly, the surge in mitotic activity is coincident with the appearance of RER-rich cells and subsequently recognisable sheath cells. This hypothesis would indicate that components of the neural lamella can be synthesised by both invasive haemocytes and sheath cells, these representing derivatives of the same stem cell. Against the hypothesis that invasive blood cells (gc-cells) become RER-rich cells, and subsequently sheath cells, is the earlier observation that perturbation of blood cell function and the reduction in the gc-cell number did not prevent the appearance of the RER-rich cells (Treherne et al. 1988). Alternatively, therefore, we may be looking at a previously unrecognised class of reactive glial cell.

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