AN ELECTRON-MICROSCOPE STUDY OF INTRACEREBRAL INFECTION OF MICE WITH LOW-VIRULENCE *BORDETELLA PERTUSSIS*

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PLATES X-XIII

THE use of the intracerebral route for the infection of mice with *Bordetella pertussis* was introduced by Kendrick *et al.* (1947) and was later adopted for the potency testing of *Bord. pertussis* vaccines. The similarities between the site of infection in the mouse brain and the more usual site of infection in man are the ciliated ependymal cells that line the ventricles of the brain and the ciliated epithelial cells of the respiratory tract. Both sites of infection, it has been suggested, are extracellular, the organisms being attached to the cilia of the respective epithelial cells (Rich, 1932; Berenbaum, Ungar and Stevens, 1960; Iida *et al.*, 1966). In the brain, histological evidence for this was provided from a study of mice dying of infection (bacterial counts 10^7 – 10^8 viable organisms per mouse brain) when small colonies of bacteria were found on the surface of ependymal cells (Iida *et al.*; Hopewell and Adams, 1970). Adams (1970) confirmed that strains of *Bord. pertussis* can be divided into two groups, (i) those of high virulence and (ii) those of low virulence, on the basis of their behaviour when injected by the intracerebral route into mice. Low-virulence strains are much more common and produce transient infections with initial inocula as large as 10^6 viable organisms, whereas the relatively rare high-virulence strains are lethal in inocula of as little as 10^3 viable organisms. The eradication of low-virulence strains of *Bord. pertussis* from the ventricles seems to be effected by local phagocytes, the neuroglia (Adams and Hopewell, 1970), although the exact nature of all of the cell-types involved is still not clear (Hopewell and Adams, 1970).

The purpose of the present electron-microscope investigation was to learn more about the site of infection on ependymal cells and also to gain a better understanding of the cell-types involved in the eradication of low-virulence *Bord. pertussis* strains from the brain.

MATERIALS AND METHODS

Under light ether anaesthesia young adult N.I.H. mice of 14–18 g were challenged intracerebrally with 0.025 ml of a suspension of a low-virulence strain of *Bord. pertussis* (B1772) in 1 per cent. (w/v) casamino acid (Difco "Technical"). The estimated bacterial inoculum was approximately 10^6 viable organisms per mouse brain. A group of similar uninfected animals was used as controls.

At intervals of 1, 2, 3 and 6 days after infection, groups of two or three mice were killed by cardiac perfusion with a 4 per cent. solution of chilled glutaraldehyde in phosphate buffer pH 7.2. After perfusion for a period of 10 min., the brains were dissected out and fixed for a further 24 hr by immersion in the glutaraldehyde solution at 4°C. The part of the cerebrum containing lateral ventricles was then cut coronally into approximately 1-mm slices and, from these, small pieces of tissue containing lateral ventricles were taken. The position of the ventricles was well defined in most of the infected brains as a result of slight internal hydrocephalus. The selected blocks of brain were post-fixed in Palade's osmium tetroxide, dehydrated, impregnated with Araldite mixture, held at room temperature for 48 hr and then embedded in Araldite. The blocks were orientated from thick sections (c. 1 μ m) stained

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INTRACEREBRAL *BORDETELLA PERTUSSIS* IN MICE

FIG. 1.—Mouse brain 2 days after infection. Individual *Bordetella pertussis* organisms (b) occurring between microvilli (m) on the surface of an ependymal cell. Electron micrograph (EM). $\times 22,500$.

FIG. 2.—Two days after infection. A single bacterial cell (b) is enclosed by microvilli (m). Cilia (c) are seen in transverse section. EM. $\times 45,000$.

INTRACEREBRAL *BORDETELLA PERTUSSIS* IN MICE

FIG. 3.—Two days after infection. A small process (p) from an ependymal cell protrudes into the ventricular space. The absence of organelles is characteristic of these processes. EM. $\times 11,375$.

FIG. 4.—Two days after infection. A large process from an ependymal cell lies in the ventricular space. The membrane-bounded (phagocytic) vacuoles (v) contain mainly normal bacteria (b) although some show signs of degeneration (d). EM. $\times 14,000$.

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FIG. 5.—Mouse brain 6 days after infection. The large phagocytic vacuoles (v) are now empty apart from the remains of dead bacteria (k). EM. $\times 28,000$.

FIG. 6.—Two days after infection. A process from a neuroglial cell (n) lies between endepymal cells (E) and contains numerous strands of endoplasmic reticulum with contents denser than the surrounding cytoplasm. The cell labelled L is thought to be a polymorphonuclear leucocyte. EM. $\times 8750$.

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FIG. 7.—Three days after infection. Neuroglial cells (N) separate ependymal cells (E) and protrude into the ventricular space. The cell in the ventricle has ribosomes attached to the outer nuclear membrane. EM. $\times 4200$.

FIG. 8.—Two days after infection. A large neuroglial cell process within the ventricular space contains numerous bacteria. EM. $\times 14,000$.

with Azur II and then thin sections were cut out and mounted on unsupported grids and stained with a saturated solution of uranyl acetate in 50 per cent. ethanol and lead citrate or lead citrate only. Electronmicrographs were taken with an AEI 6B electron microscope.

RESULTS

Observations on the site of infection

During the first 3 days after infection, when the bacterial counts per mouse brain were highest (Adams and Hopewell, 1970), bacteria were to be found, very unevenly distributed, either singly or in small groups in close proximity to ependymal cells in the ventricular space. The bacteria appeared to be held between the intermingled microvilli of the ependymal cells (figs. 1 and 2). The bacteria were capsulate and usually oval in cross-section, with a mean diameter of $0.48\ \mu\text{m}$, although some had become very elongated. There was evidence of bacterial multiplication at the site of infection. There was no evidence to support previously published views that the cilia *per se* were the site of attachment for the microbes.

Observed changes in ependymal cells

As early as 1 day after infection, ependymal cells put out processes into the ventricular space. The protoplasm in these processes is of uniform electron density and contains few if any organelles (fig. 3). The processes do, however, contain membrane-bounded vacuoles of which the larger ones contain a variable number of bacteria (fig. 4). During the first 3 days after infection the majority of bacteria appear morphologically normal, although a few are obviously degenerating. The vacuoles that we observed are presumably phagocytic. Six days after infection, when the bacterial count in the brain has declined, the large phagocytic vacuoles are empty apart from the remains of degenerate bacteria (fig. 5).

Observed changes in neuroglia

From 1 to 6 days after infection, marked changes were observed in the neuroglia which underlies the normally continuous ependymal cell layer. The continuity of the ependymal layer was disrupted, ependymal cells being separated by neuroglia cell processes that extended into the ventricular space (fig. 6). Sometimes the break in the continuity of the ependymal layer was large and the gap was filled by several neuroglial cells. Cells or cell processes also extended into the ventricular space (fig. 7) and occasionally they contained bacteria (fig. 8). Unlike the processes from ependymal cells, the cell processes derived from underlying glia were rich in organelles. These organelles always included endoplasmic reticulum, the contents of which were often more electron-dense than the surrounding cytoplasm (fig. 6). In these neuroglial cells there were free ribosomes, although in some cells, ribosomes were attached to the outer nuclear membrane (fig. 7). Mitochondria were often present in large numbers and they often contained vesicles (fig. 7). In other cells a few glycogen particles and fine fibrils were found.

In addition to the changes in ependymal and neuroglial cells there was also evidence of subependymal infiltration by polymorphonuclear leucocytes within the first 3 days after infection, although only small numbers were involved.

DISCUSSION

The results give firm evidence that the site of infection for *Bord. pertussis* in the mouse brain is between the microvilli of ependymal cells that line the ventricles of the brain. Nevertheless, the site of infection in the brain is still strictly comparable with the normal site of infection in the respiratory tract. The surfaces of epithelial cells in the respiratory tract have also been shown to have microvilli (Porter and Bonneville, 1963; Rhodin, 1963; Garven, 1965). There was no evidence of bacterial attachment to cilia which had previously been suggested as the site of infection (Iida *et al.*, 1966; Hopewell and Adams, 1970).

The observations also show that ependymal cells as well as the underlying glia react, after "intracerebral" infection with *Bord. pertussis*. The glial cells reach the ventricular

space by passage between ependymal cells, as was suggested previously from light-microscope evidence (Hopewell and Adams). The bacteria appear to be phagocytosed by both cell-types. Phagocytosis by neuroglia cells has been reported previously (del Rio-Hortega and Penfield, 1927; Lebowich, 1934), but there are no previous reports of phagocytosis by ependymal cells. In addition, the finding that the microbes are phagocytosed by both glial and ciliated ependymal cells demonstrates a means by which a superficially situated microbe may become parenteral and then act as an antigen.

An exact identification of the neuroglial cell-types involved is difficult because of the marked transformations undergone by reactive cells. However, the observations suggest that the reactive cells are a mixed neuroglial population of microglia and astrocytes. The presence of ribosomes on the outer nuclear membrane is a character often associated with microglia (Blakemore, 1969) whilst glycogen and microfibrils are usually linked with astrocytes (Maxwell and Kruger, 1965). These two cells are the predominant types found underlying the ependyma in rats (Blakemore).

The presence of only small amounts of glycogen in reactive astrocytes is inconsistent with the observations of Maxwell and Kruger who have found large amounts of glycogen in these cells. A possible explanation of our finding is, however, provided by the same authors who showed that the glycogen rapidly disappeared if fixation was not completed immediately after death. In our material, hydrocephalus was often associated with highly infected brains (Adams, 1968; Hopewell and Adams) and this may have impeded fixation to some extent.

As was the case in light-microscope studies (Hopewell and Adams), only a few polymorphonuclear leucocytes were to be found in highly infected brains. The observed paucity of blood-borne cells, along with the other observations presented here, support the view that a local mechanism is responsible for the eradication of low-virulence *Bord. pertussis* from the mouse brain and this confirms the earlier conclusions of Hopewell and Adams.

SUMMARY

An electron-microscope study of experimentally infected mouse brain after intracerebral challenge with a low-virulence strain of *Bordetella pertussis* has shown the site of infection to be extracellular and between the microvilli of the ependymal cells. The types of local cells reacting in response to the infection were the ependymal cells and the underlying neuroglia, the latter reaching the ventricular space between the ependymal cells. Evidence of phagocytosis by the two different cell-types was found and there was evidence of subsequent bacterial destruction within these cells.

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