Interactions of virulent and avirulent leptospires with primary cultures of renal epithelial cells

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Summary. A primary culture system for the cells of mouse renal-tubular epithelium was established and used to observe the adhesion of leptospires. Virulent strains of serovars *copenhageni* and *ballum* attached themselves to epithelial cells within 3 h of infection whereas an avirulent variant of serovar *copenhageni* did not adhere to epithelial cells at all within the experimental period of 24 h. The saprophytic *Leptospira biflexa* serovar *patoc* became attached non-specifically to inert glass surfaces as well as to the cells. The adhesion of leptospires to epithelial cells was not inhibited by homologous antibody.

Introduction

The dissemination of leptospires in the urine of carrier animals is a critical factor in the epidemiology of leptospirosis. Leptospires survive in the renal proximal tubules, multiplying and colonising the tubular epithelium. The presence of specific antibody in the urine appears not to protect the host against the establishment or maintenance of the carrier state (Lane and Faine, 1963).

A frequent requirement for the establishment of infection is the ability of the causative micro-organism to adhere to host-cell surfaces (Smith, 1977). In electronmicroscopic studies leptospires were observed in close association with the microvilli of proximal renal-tubular epithelial cells during chronic leptospirosis in hamsters (Miller and Wilson, 1967) and in sheep (Marshall, 1974). Colonisation of the renal tubules by leptospires is accompanied by their adhesion to the epithelial-cell surface.

Many workers have studied the interactions of leptospires with cultivated mammalian cells in attempts to relate cytotoxicity or adhesion of leptospires to pathogenesis. Leptospires caused visible cell damage in cultures of kidney cell lines (Finn and Jenkin, 1973) and in fibroblastic cell lines (Miller *et al.*, 1970; Yam *et al.*, 1970) in which adhesion to cell surfaces preceded cell damage (Vinh *et al.*, 1984). However cytotoxicity was restricted neither to virulent strains nor to pathogenic serovars, saprophytic serovars being equally cytotoxic. This contrasts strikingly with the lack of cell damage observed by electronmicroscopy in

epithelial cells of carrier animals (Miller and Wilson, 1967; Sterling and Thiermann, 1981). Thus it is difficult to assess the relevance of the in-vitro observations described above to pathogenesis of the chronic carrier state.

We wished to examine whether the ability of leptospires to adhere to renal epithelial cells might be related to virulence, to the ability to establish the carrier state, or to both. In this paper we report the interactions between leptospires and primary cell cultures of renal tubular-epithelial cells. Such cell cultures bear a closer resemblance than fibroblastic cell lines to the environment of leptospires in natural carriers.

Materials and methods

Leptospires

Leptospira interrogans serovar *copenhageni* (45H) and its avirulent variant (45S) were described previously (Faine and van der Hoeden, 1964), as were serovar *ballum* (17) and *L. biflexa* serovar *patoc* (41) (Adler and Faine, 1977). Leptospires were grown in EMJH medium with added pyruvate (Johnson *et al.*, 1973) for 4–7 days at 30°C and counted by means of a Thoma bacterial counting chamber.

Preparation of epithelial-cell monolayers

Primary cell cultures of mouse renal-tubular epithelium were used because a renal carrier state is known to follow infection with leptospires in mice.

In each experiment, three Balb/c mice were killed by cervical dislocation, the kidneys removed aseptically and the renal cortices dissected out and diced finely. The

cortical tissue was then mechanically minced on the surface of a 250- μ m stainless steel mesh by means of a stainless steel spoon; fragments were then washed through with tissue culture medium 199, pH 7.4, (M199; Commonwealth Serum Laboratories (CSL), Melbourne) containing (/ml) sodium bicarbonate 0.1%, penicillin G 100 units, streptomycin sulphate 100 μ g and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (Hepes) 0.34 mg. The fragments of tubule in the filtrate were collected and further disrupted by mincing and washing through 184- μ m stainless steel mesh. In the final step the crude preparation of tubule fragments was washed through 103- μ m stainless steel mesh, which retained the tubule fragments but not unwanted single cells, glomeruli or cell debris. Tubule fragments were collected and resuspended in 12.0 ml of M199 containing fetal calf serum (fcs; CSL) 20%. One-ml volumes were dispensed into the wells of a tissue culture tray (Costar, Flow Laboratories) containing glass coverslips (4 $\times$ 5 mm), and incubated at 37°C in CO₂ 5% for 7–10 days.

Stains

Alkaline phosphatase stain. Epithelial cell monolayers were washed thoroughly with phosphate-buffered saline pH 7.2 (PBS), then fixed in acetone for 30 min at 4°C. Fixed monolayers were stained by the method of Gomori as described by Bancroft (1967) and examined microscopically under bright field illumination with a Zeiss IM35 microscope.

Indirect immunofluorescent staining for actin filaments. Epithelial cell monolayers were fixed and stained as described by Vinh *et al.* (1984) with rabbit anti-actin serum and examined with a Zeiss IM35 microscope equipped with epifluorescence illumination.

Co-incubation of leptospires and epithelial cell monolayers.

Five-day-old cultures of leptospires were centrifuged at 27100 *g* for 10 min and the supernates discarded. Pelleted leptospires were washed twice with M199 without antibiotics (M199w) and resuspended in M199w with fcs 20% at a concentration of 5×10^8 leptospires/ml for phase-contrast and immunofluorescence microscopy or 8×10^8 leptospires/ml for scanning electronmicroscopy (SEM).

Seven-day-old epithelial-cell monolayers were washed three times with M199w before being seeded with 1.0 ml of leptospiral suspension. Infected cell monolayers were incubated at 37°C in CO₂ 5% for 1, 2, 3, 6 or 24 h; at the end of the experiment unattached leptospires were removed by washing the coverslips three times with PBS.

The effect of antibody on adhesion of leptospires to epithelial cells was examined by adding a subagglutinating concentration of homologous mouse antiserum to the leptospiral suspension immediately before it was used to seed cell monolayers. Titres of mouse antisera were determined by the microscopic agglutination test (MAT; Faine, 1982) modified to read a zero end-point. A dilution

of antiserum twice that of the MAT titre was considered subagglutinating.

Preparation of antisera

Homologous rabbit antisera were prepared as described by Adler and Faine (1977). Homologous mouse antisera were prepared in Balb/c mice by intraperitoneal inoculation of 0.5 ml of leptospiral culture (5×10^8 leptospires/ml) grown in protein-free polysorbate 60/40 medium (Christopher *et al.*, 1982). Mice received a similar booster injection after 7–10 days and were bled by cardiac puncture 7 days later.

Phase-contrast microscopy

Coverslips with islands of epithelial cells were inverted on to a microscope slide and the edges sealed with wax. Direct observations of the interaction of epithelial cells and leptospires were made by phase-contrast microscopy with a Zeiss IM35 inverted microscope.

Immunofluorescence microscopy

Infected epithelial-cell monolayers were fixed with 1% neutral buffered formalin for 1 h at room temperature. Coverslips were stained by indirect immunofluorescence as described by Vinh *et al.* (1984) and examined with a Zeiss IM35 microscope equipped with epifluorescence illumination.

Scanning electronmicroscopy

Monolayers were fixed in glutaraldehyde 4% in cacodylate buffer, pH 7.4, for 1 h at room temperature. They were then given four washes in cacodylate buffer, pH 7.4, and two in distilled water before being post fixed in osmium tetroxide 2% in distilled water for 30 min at room temperature. The coverslips were then washed four times with distilled water and dehydrated first in a series of increasing ethanol concentrations and then by two further treatments with absolute ethanol, each for 15 min, and two treatments with 100% dry ethanol. All reagents had been filtered through a 0.22- μ m membrane (Millipore) before use. The coverslips were dried in a critical point drying apparatus (Samdri, USA) and attached to aluminium stubs before being coated with gold by means of a Polaron 5000 Sputter Coater. Coverslips were then observed in a Cambridge 50 scanning electron microscope at 20 kv.

Results

Preliminary cell-culture experiments

Mouse renal tubule fragments, prepared as described, adhered to glass coverslips within 4 days. Epithelial cells grew radially in islands migrating from adherent fragments. Optimal numbers of

Fig. 1. Scanning electronmicrograph of epithelial cell growth derived from tubule fragment. Original site of tubule fragment (A). Bar = 200 μm (top) and 20 μm (bottom).

epithelial cells for co-incubation experiments were found after 7–10 days in primary culture. After 10 days the cells began to deteriorate and slough off the coverslip.

The epithelial nature of the cell population was confirmed by histochemical staining for alkaline phosphatase. Cells reacted strongly after staining by the modified Gomori technique, indicating alkaline phosphatase activity. The absence of fibroblasts from the cultures were established by means of an immunofluorescent anti-actin stain.

Scanning electronmicroscopy of the islands showed variation in the size and morphology of cells (fig. 1). The cells adjacent to tubule fragments were closely packed and larger than those at the periphery of the island, which were small and slightly elongated with many filipodia. Microvilli were observed on the surface of both epithelial-cell types.

Scanning electronmicroscopy (SEM)

Attachment of the pathogenic *L. interrogans* serovar *copenhageni* (45H), its avirulent form (45S), serovar *ballum* and the non-pathogenic *L. biflexa*

serovar *patoc* to renal tubular epithelial cells was investigated.

The virulent 45H strain of serovar *copenhageni* adhered to epithelial cells in very small numbers over a period of 24 h. Leptospires attached themselves in discrete clumps to the cell surface and especially to the edges of peripheral epithelial cells (fig. 2). Few leptospires were observed to adhere to the large epithelial cells where filipodia and intercellular spaces were absent. The amount of adhesion was not proportional to the density of microvilli, but leptospires were rarely observed to adhere to epithelial cells without microvilli. The avirulent 45S strain of serovar *copenhageni* did not adhere to epithelial cells at all, even after co-incubation for 24 h.

Serovar *ballum* (fig. 3) adhered to the cell surface in clumps within 2 h. The number of leptospires attaching appeared to increase with length of incubation, reaching a maximum after *c.* 6 h.

In contrast serovar *patoc* attached indiscriminately to epithelial cells and to the glass coverslip. The leptospires attached “end-on” to the epithelial cells with only the occasional organism lying flat on the cell surface (fig. 3).

Fig. 2. Scanning electronmicrograph of epithelial cells 24 h after infection with virulent serovar *copenhageni* (45H). Bar = 4 μ m.

Fig. 3. Scanning electronmicrograph showing leptospires of serovar *ballum* attached to epithelial cells (top) and of serovar *patoc* attached non-specifically to glass surface as well as to cells (bottom). Bar = 10 μ m.

Fig. 4. Phase-contrast wet mount of leptospires of serovar *ballum* attached to epithelial-cell surface 2.5 h after infection. Bar = 20 μ m.

No evidence of epithelial-cell damage attributable to the presence of any of the leptospiral strains was observed.

Phase-contrast and immunofluorescence microscopy

Phase-contrast and immunofluorescence microscopy were used to investigate attachment of the two pathogens, serovar *copenhageni* (45H) and serovar *ballum*, and the non-pathogenic serovar *patoc*, to epithelial cells to compare with the observations made by SEM. The degree of adhesion to epithelial cells and the patterns of adhesion for serovars *ballum*, *copenhageni* and *patoc* were similar to those observed by SEM (fig. 4).

Effect of homologous antibody

The effect of homologous antibody prepared in mice on the attachment of leptospires to renal tubular epithelial cells was examined by SEM. A subagglutinating concentration of antibody had no observable effect on the attachment of serovar *ballum* or the avirulent strain 45S of serovar *copenhageni*. However, higher concentrations of antibody resulted in the attachment of large agglutinated clumps of leptospires to the glass coverslip

(fig. 5). In contrast, a subagglutinating concentration of homologous antibody enhanced the attachment of the virulent strain 45H of serovar *copenhageni* to epithelial cells. Leptospires were still observed to attach, in clumps, particularly to peripheral epithelial cells but in numbers significantly greater than those observed in the absence of antibody, especially during the first 3 h after inoculation. A subagglutinating concentration of antibody also enhanced the attachment of the non-pathogenic serovar *patoc* to the epithelial cells and to the glass coverslip.

Normal mouse serum was invariably without effect on the adhesion of leptospires. No cytopathic effect (CPE) on the epithelial cells was observed within 24 h when antiserum was present.

Discussion

Adhesion to various host tissues appears to be one of the many complex factors in microbial virulence. Tissue culture has proved useful in the study of host-parasite interactions because it is independent of host immunological factors. In this paper the interactions of leptospires with epithelial cells obtained by primary culture of mouse proximal tubule explants are reported.

Fig. 5. Scanning electronmicrograph of leptospires of virulent serovar *copenhageni* (top) and *patoc* (bottom) incubated for 1 h with epithelial cells in the presence of subagglutinating concentrations of homologous mouse antiserum. Bar = 20 μ m.

Unlike 72-h experiments with leptospires in fibroblast cultures (Vinh *et al.*, 1984), our 24-h experiments in renal tubular epithelial cells showed no cytopathic effects attributable to the presence of virulent organisms. This is consistent with the lack of cell damage observed in epithelial cells of carrier animals (Miller and Wilson, 1967; Sterling and Thiermann, 1981) and other epithelial-cell cultures (Miller *et al.*, 1966).

Two morphologically different cell types, both epithelial, were observed growing radially from the proximal tubule explants. The peripheral cells appeared to represent younger cells in an earlier stage of growth. Virulent leptospires from serovars *copenhageni* and *ballum* adhered particularly to the peripheral epithelial cells for reasons that are unclear, as is the significance of these observations for the renal carrier state.

A comparison of adhesion of virulent and avirulent strains of serovar *copenhageni* to epithelial cells suggests a correlation between virulence and adhesion. Vinh *et al.* (1984) demonstrated that avirulent *copenhageni* adhered less than virulent *copenhageni* to cultured L-cells. Moreover, Tsuchimoto *et al.* (1984) reported that virulent strains of serovars *copenhageni*, *canicola* and *pomona* readily attached to MDCK cells whereas avirulent strains of the same serovars attached poorly. Fitzgerald *et al.* (1977) found that large numbers of *Treponema pallidum* attached to cultured cells (epithelial and fibroblastic), but non-pathogenic treponemes failed to do so.

In view of the suggestion of a relation between virulence and adhesion in pathogenic leptospires, the non-pathogenic serovar *patoc* would not be expected to attach to epithelial cells *in vitro*. In this study, however, and throughout the literature, serovar *patoc* has been reported to attach in large numbers to cultured cell lines (Kefford, 1983; Vinh *et al.*, 1984); this attachment may not be specific as the organisms also attach in large numbers to glass and plastic surfaces (Kefford and Marshall, 1984; Tsuchimoto *et al.*, 1984; Vinh *et al.*, 1984). Bacteria that adhere to inert surfaces do so non-specifically, by mechanisms that may also contribute to the adhesion of bacteria to eukaryotic cells (Marshall and Bitton, 1980). In such instances cultured epithelial or fibroblastic cell lines may merely act as another surface to which leptospires attach non-specifically (Kefford, 1983). The attachment of saprophytic strains to cells is therefore irrelevant to studies of pathogenesis.

Specific antibody has been reported both to inhibit and to enhance leptospiral adhesion to host-

cell surfaces *in vitro*, but the reasons for the various effects are unclear. Tsuchimoto *et al.* (1984) observed a decrease in the numbers of leptospires attaching to MDCK cells with increasing concentrations of Fab fragments of homologous IgG. In contrast Vinh *et al.* (1984) demonstrated enhancement of leptospiral adhesion to fibroblasts by subagglutinating amounts of homologous antibody.

In the present study the enhancing effect of specific antibody on the adhesion of leptospires to epithelial cells depended on antibody concentration. When agglutinating amounts of homologous antibody were used, all strains of leptospires aggregated into clumps and attached non-specifically and in large numbers to inert surfaces, reducing the numbers of organisms available for attachment to epithelial cells. When subagglutinating concentrations of homologous antibody were used to avoid this problem, they had no effect on the adhesion of serovar *ballum* or serovar *copenhageni* (avirulent strain) but enhanced specific adhesion of serovar *copenhageni* (virulent strain) and non-specific adhesion of serovar *patoc*.

In the carrier animal, antibody does not prevent the colonisation of the proximal tubules or shedding of organisms from the kidney (Faine, 1962). However, carrier animals frequently shed leptospires intermittently and this may in part reflect antibody inhibition or enhancement of leptospiral adhesion in the proximal tubules. Our observations suggest that varying concentrations of specific antibody can modulate adhesion of leptospires to the tubular epithelium.

In this paper we have established a culture system for renal epithelial cells, and shown that ability to adhere to these cells may be a necessary property of virulent leptospires, reflecting their behaviour *in vivo*. This system would provide a useful model for further study of the pathogenesis of leptospirosis if quantitative methods for measuring adhesion were to be developed.

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