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Effect of respiratory syncytial virus infection on binding of *Neisseria meningitidis* and *Haemophilus influenzae* type b to a human epithelial cell line (HEp-2)

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SUMMARY

It has been suggested that individuals might be more readily colonized with bacteria that cause meningitis through enhanced binding of the bacteria to virus-infected epithelial cells. As respiratory syncytial virus (RSV) affects infants and children in the age group also susceptible to bacterial meningitis, we tested the hypothesis that infection of HEp-2 cells by RSV might enhance binding of *Neisseria meningitidis* or *Haemophilus influenzae* type b (Hib). Attachment of fluorescein-labelled bacteria to HEp-2 cells was measured by flow cytometry, and RSV-infected cells bound significantly more meningococci ($P < 0{\cdot}001$) and Hib ($P < 0{\cdot}01$) than uninfected cells. Although the isolates expressed different antigenic characteristics (3 meningococci and 5 Hib), all showed a similar pattern of binding. The results are discussed with reference to the methods used for detection of bacterial binding and to interactions that might explain the increased binding to RSV-infected cells.

INTRODUCTION

Clinical, epidemiological and experimental evidence indicates that virus infections can be predisposing factors for invasive bacterial diseases and carriage of potentially pathogenic bacteria [1–6]. Factors considered to contribute to this effect include: immune suppression [7–9]; diminished phagocytosis by polymorphonuclear leucocytes [10]; local oedema formation and tissue injury; loss of mucociliary function and decreased bacterial clearance [11]; formation of exudates that enhance bacterial growth [7]; and increased bacterial binding to virus-infected cells [7]. Most investigators have studied associations of influenza virus and bacteria such as *Streptococcus pneumoniae* [12], *Staphylococcus aureus* [13] and *Haemophilus influenzae* [14, 15]. Coincident upper respiratory tract infections with viruses or mycoplasma have been reported to be risk factors for epidemic disease due to group A *Neisseria meningitidis* [16]; and there is recent epidemiological evidence for an increase in meningococcal disease following epidemics of influenza A [17].

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Human respiratory syncytial virus (RSV) is a ubiquitous respiratory tract pathogen which infects 50% of children in their first year of life and virtually all by the age of 2 years [18]. Much of the invasive disease due to meningococci and *H. influenzae* type b (Hib) occurs in this age group [19].

Although animal models have been used to study disease caused by meningococci and Hib, we chose to use a human epithelial cell line which expresses surface antigens, H and Lewis^b postulated to be involved in bacterial binding [20]. HEp-2, a non-ciliated human epithelial cell line, can be infected with RSV, and uninfected cells have been used for bacterial attachment studies [21, 22]. In the present study we tested the hypothesis that HEp-2 cells infected with RSV might bind meningococci or Hib to a greater extent than uninfected cells.

MATERIALS AND METHODS

Cells

HEp-2 cells (Flow Laboratories) were used between passages 400 and 420 for the binding assays. Cell growth medium (GM) consisted of Eagle's minimal essential medium (Gibco) supplemented with 10% foetal calf serum (FCS) (Gibco), $NaHCO_3$ (0·85 g/l), L-glutamine (2 mM), streptomycin (200 μg/ml) and penicillin (100 IU/ml). Maintenance medium (MM) contained all the constituents of GM but only 1% FCS.

Virus

RSV (Edinburgh strain) [23] was used at passages 5–7 to infect the HEp-2 cells. Stock aliquots of supernatant fluid from infected cell lysates were stored at −70 °C and contained from 1 to 5×10^6 plaque forming units (p.f.u.)/ml.

Bacteria

Three strains of meningococci were used: C:2b:P1.2; NG:-:P1.9; Y:14:P1.2. Pilate and non-pilate isolates of Y:14:P1.2 were tested. Hib isolates from Icelandic children were kindly provided by Dr K. Jónsdottir, University of Iceland (Table 1). Serogroup, serotype and subtype of the meningococcal isolates were determined by Dr R. J. Fallon, Meningococcus Reference Laboratory (Scotland). Cultures were prepared by reconstituting the lyophilized bacteria in distilled water; meningococci were grown on modified New York City (MNYC) medium [24] and Hib on heated blood agar. For some experiments meningococci were grown on heated blood agar or GC agar (Difco, UK) containing 10% lysed horse blood, 2·5% yeast dialysate and 10% glucose to examine the effect of growth medium on attachment. Bacteria were grown overnight at 37 °C in 5–10% CO_2 in air. Dulbecco's phosphate buffered saline solution A (DPBSA) was used for washing the bacteria and the cells.

The bacteria were harvested, washed three times in DPBSA by centrifugation at 2500 g for 15 min and resuspended in MM without antibiotics by vigorous pipetting to disperse clumps. The bacterial concentration was determined by measuring optical density (OD) at 540 nm. The linear relationship between OD reading and total count was determined for each strain.

Table 1. *Isolates of type b* Haemophilus influenzae *examined*

Strain no.	Source	Enzyme type	Biotype	OMP type	LPS type
14	Edinburgh	—	—	—	—
20	Reykjavik	4b	I	NT*	1
21	Reykjavik	1	I	2	9
25	Reykjavik	12	I	1	1
29	Keflavik	1	—	—	—

* NT, non-typable.

Binding of meningococci to HEp-2 cells in suspension

Overnight monolayer cultures of HEp-2 cells in culture flasks (25 cm^3) were infected with RSV at different multiplicities of infection (MOI) ranging from 0·001 to 1·0 p.f.u./cell. Virus (1 ml in MM) was adsorbed for 1 h, replaced with 10 ml MM and incubated overnight at 37 °C. Cultures were rinsed twice with DPBSA, then ethylenediaminetetraacetic acid (EDTA) (0·05%) was applied, 1 ml per flask at 37 °C for 5–10 min to produce a cell suspension. MM (5 ml) was then added to the cells to counteract EDTA activity. After centrifugation at 460 g for 7 min, the cells were resuspended in MM without antibiotics, counted and adjusted to 1×10^6 cells/ml. The cell suspensions were gently rotated at 37 °C whilst the bacteria were prepared. The time from end of viral adsorption to addition of bacteria was 24 h (±1 h).

The bacterial suspensions were labelled with fluorescein isothiocyanate (FITC) (Sigma) freshly prepared as a 0·4% solution in NaH_2CO_3 (0·05 M) and NaCl (0·1 M) (pH 9·2). The washed bacterial pellet obtained from two culture plates was suspended in 2 ml of the FITC solution by gentle shaking at 37 °C for 20 min [25]. FITC-labelled bacteria were washed three times in DPBSA and resuspended in MM without antibiotics. The piliate isolate was incubated at 37 °C for 2 h to allow pili to regenerate. The concentration of the suspension was determined by OD at 540 nm and adjusted to provide a range of ratios of bacteria per cell. The bacterial suspensions (200 µl) were incubated with equal volumes of HEp-2 cells at 37 °C at 60 rpm in an orbital incubator (Gallenkamp). At the end of each incubation period, the cells were washed three times by centrifugation at 460 g for 7 min. The cells were resuspended in 200 µl of DPBS and fixed with 100 µl of 10% buffered paraformaldehyde (Sigma). The samples were kept in the dark at 4 °C until analysed within 3 days of the assay.

Analysis of binding by flow cytometry

Each sample was analysed by flow cytometry with an EPICS-C (Coulter Electronics, Luton, UK) equipped with a 5-watt laser with a power output of 200 mw at 488 nm. The cells were selected from a display of forward angle light scatter (size) versus 90° light scatter (granularity) by means of a bitmap. The bitmap included the main population of the cells excluding unbound bacteria, debris and clumps from further analysis. The percentage of cells with fluorescence greater than background level was recorded on a one-parameter histogram (H-%) measuring fluorescence on a logarithmic scale. The mean fluorescence channel values for the positive cells were obtained from a one-parameter histogram

measuring fluorescence on a linear scale. After subjecting the H-% histograms to 'Immunoanalysis' (Coulter), a computer program that subtracts the values of the control population from the test population at each channel of the two H-% histograms, the binding index (BI) of each sample was calculated by multiplying the percentage of fluorescent cells by the mean fluorescence.

RSV-infected cells were reacted with mouse monoclonal antibody to RSV-F protein (kindly supplied by Professor P. J. Watt, Southampton University) for 30 min at 37 °C. After washing, 20 μl of FITC-labelled antimouse IgG antibody (Fab fragment, adsorbed with human serum protein) (Sigma) was added and incubated for 30 min. The cells were washed and fixed in paraformaldehyde and the proportion of cells binding the monoclonal anti-F antibody was determined by flow cytometry.

Binding of H. influenzae *to HEp-2 cells*

The same method was used to investigate the binding of *H. influenzae* to HEp-2 cells infected with RSV at MOI 1·0. The bacteria (Table 1) were grown on boiled-blood agar medium for 48 h. FITC-labelled bacteria were incubated with cells in ratios of 50, 200 and 800 bacteria per cell for 2 h, the incubation period recommended by St Geme and Falkow [26]. The cells were analysed by flow cytometry as described above.

Statistical methods

The results were analysed with the statistical package 'Minitab' using multiple regression on the logarithms of the BIs of the samples. For experiments with meningococci, dummy variables were included to adjust for daily variations and different ratios of viruses and bacteria/cell. The coefficients from this analysis were used to estimate binding relative to that found with 10 bacteria/uninfected cell by taking antilogarithms. Examination of the distribution of residuals from the fitted models suggested that the logarithmic transformation had given an acceptable fit to a normal distribution. A sign test was used to test for consistency of excess binding of *H. influenzae* to RSV-infected cells in repeated experiments.

RESULTS

Detection of RSV-infected cells

The percentage of RSV-infected cells at 24 h post inoculation for each MOI was determined. This ranged from $< 10\%$ (MOI = 0·001) to $> 80\%$ (MOI = 1) at the time of the attachment assay. No cytopathic effect was noted at this stage.

Attachment of meningococci to cells in suspension assessed by flow cytometry

The effect of RSV infection on binding of meningococci to HEp-2 cells is shown in Figs 1 and 2. The left-hand peak in the figures represents fluorescence recorded with uninfected cells following incubation with 320 bacteria/cell. The right-hand peak represents fluorescence observed with cells infected with MOI of 0·1 (Fig. 1) and 1·0 (Fig. 2) following incubation with 320 bacteria/cell.

Table 2 summarizes the results obtained by flow cytometry assays to assess the effect of viral infection at various MOI on binding of bacteria. The results for 11

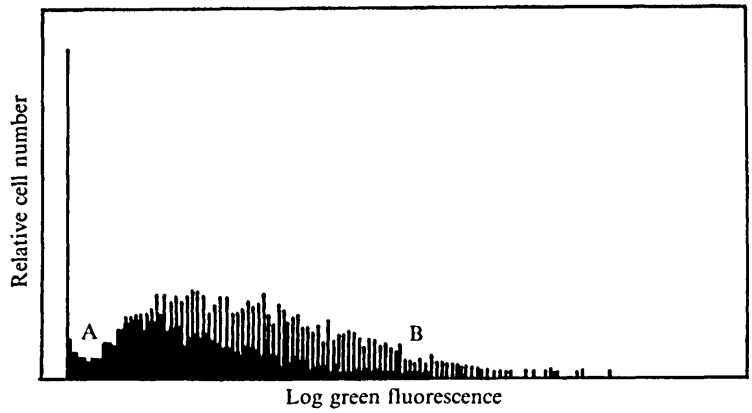

Fig. 1. Binding of FITC-labelled *N. meningitidis* to HEp-2 cells infected with RSV (MOI 0·1) (B) and uninfected cells (A).

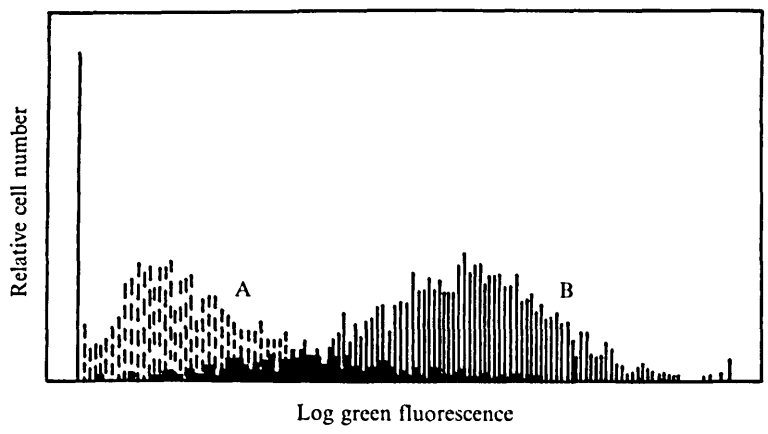

Fig. 2. Binding of FITC-labelled *N. meningitidis* to HEp-2 cells infected with RSV (MOI 1·0) (B) and uninfected cells (A).

Table 2. *Effects of infection of HEp-2 cells with RSV on binding of* N. meningitidis

Estimated binding indices from multiple regression

Bacteria added per cell	Virus added (MOI) 0	0·001	0·01	0·1	1·0
10	1*	0·60	0·69	1·25	ND†
20	0·79	0·89	1·23	1·02	1·17
40	1·94	1·23	1·25	2·39	2·13
80	3·16	1·90	3·23	3·31	4·07
160	5·75	3·80	4·67	7·76	12·88
320	10·00	5·88	11·48	19·05	33·11
640	13·18	ND	ND	28·18	52·48
1000	22·38	ND	ND	56·23	112·20

* Mean for MOI = 0 and 10 bacteria per cell taken as 1.
† ND, not done.

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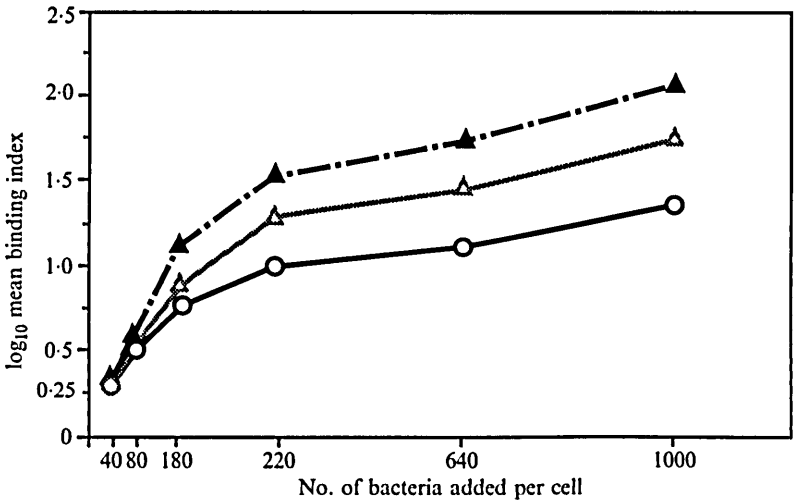

Fig. 3. Effect of infection of HEp-2 cells with RSV at MOI of 0·1 (△) and 1·0 (▲) on binding of *Neisseria meningitidis* to the cells. No virus added (○).

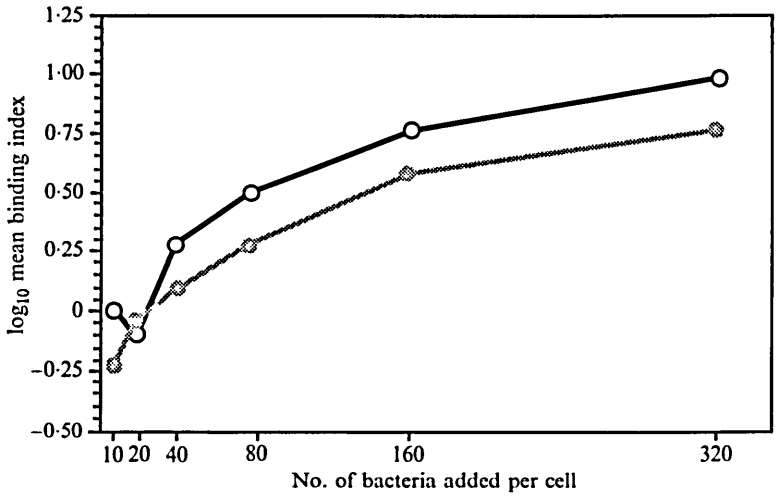

Fig. 4. Effect of infection of HEp-2 cells with RSV at MOI of 0·001 (●) on binding of *Neisseria meningitidis* to the cells. No virus added (○).

assays were combined using analysis of variance and Table 2 shows the derived estimate of the ratios of the binding index obtained with 10 bacteria/uninfected cell taken as 1. The results indicate that prior infection of the cells with RSV at MOI of 0·1 and 1·0 enhanced the binding of *N. meningitidis* at ratios > 80 bacteria/cell as illustrated in Fig. 3 ($F(2{\cdot}51) = 37{\cdot}37$ $P < 0{\cdot}001$).

At ratios of 10–20 bacteria/cell, the presence of virus infection at any level did not change the binding level significantly. The cells infected with an MOI of 0·001 demonstrated decreased bacterial binding compared with uninfected cells; ($F(1{\cdot}45) = 5{\cdot}48$, $P < 0{\cdot}05$) (Fig. 4).

The figures and Table 2 were obtained with one strain (C:2b:P1.2) of meningococcus; however, similar results were found with other strains which

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Table 3. *Effect of infection of HEp-2 cells with RSV (MOI = 1) on binding of* H. influenzae *strain H14*

	Ratio of binding of strain H14 to HEp-2 cells infected/uninfected		
	Bacteria/cell		
Experiments	80	160	320
1	ND	ND*	1·45
2	1·0	ND	2·04
3	1·0	0·99	2·14
4	0·72	1·92	1·49
5	1·64	0·69	1·0
6	2·55	2·77	4·09
7	1·52	2·4	2·41
8	1·19	1·02	1·33
9	1·02	1·04	1·05
10	1·41	1·01	1·04

* ND, not done.

expressed different serogroup, serotype and subtype surface antigens. Growth on heated blood agar or GC agar did not alter the pattern of binding obtained when the bacteria were grown on NYC medium. Presence of pili demonstrated by a haemagglutination assay had no effect on the binding pattern.

Attachment of H. influenzae *to cells in suspension*

Cells infected with RSV at MOI 1·0 bound significantly more Hib than uninfected cells. Although the effect varied widely between experiments, the mean of log differences was positive in all 10 experiments with strain H14 ($P < 0·01$, sign test). RSV infection of the cells enhanced bacterial binding at all the ratios of bacteria to cell tested (Table 3). In 7 additional assays, 4 strains of *Haemophilus* sp. with different antigenic and biotype characteristics showed a similar increase in binding to RSV-infected cells compared with uninfected cells at each bacterial:cell ratio tested.

DISCUSSION

Increased susceptibility to secondary bacterial disease following viral infection has been recognized for many years [27]. Among the factors suggested to contribute to susceptibility to secondary infections, the enhancement of bacterial binding to virus-infected cells might be an important initial step.

In initial experiments, binding of meningococci to HEp-2 cells and RSV-infected cells was assessed by light microscopy. The method was not used for quantitative studies because of wide variation in the counts obtained (data not shown) and the time needed for the assay. The experiments were, however, helpful to demonstrate binding of the bacteria to the cells and to determine optimal incubation periods before cytotoxic effects occurred. These effects might be the direct effect of the bound bacteria [21] or of the release of lipopolysaccharide from the bacteria [28]. Although increased binding of bacteria to RSV-infected cells was observed with this technique, results were not reliably reproducible. In our studies, the measurement of fluorescein-labelled bacteria on the surface of cells by

flow cytometry is a more sensitive and precise method than microscopy. More cells can be counted in a short period of time and there is no subjectivity in the values recorded.

The finding that in the cell samples with $< 10\%$ infected cells (MOI 0·001) meningococcal binding is significantly decreased compared with uninfected cells was not expected (Fig. 4). The RSV-infected cells might be secreting a soluble factor such as interferon which results in reduced bacterial binding to neighbouring uninfected cells in the sample. Compared with controls, total bacterial binding was not significantly altered for the samples containing 20–30% infected cells (MOI 0·01). This might reflect the mixed population of infected cells and uninfected cells.

Enhanced binding of meningococci and haemophilus to cells infected with RSV at MOI $\geqslant$ 0·1 p.f.u./ml 24 h previously suggests four possibilities. RSV infection might enhance the expression of existing receptor(s) for the bacteria. It might induce expression of a new cell receptor. The filaments observed on the surface of RSV-infected cells might trap the bacteria [29]. The F or G viral glycoproteins expressed on the infected cells might act as additional receptors for the bacteria. Because Hep-2 cells infected with RSV express viral glycoproteins F and G on their surface at the time used in these experiments, the last hypothesis is currently under investigation.

The results of this study suggest virus infection might be a predisposing factor for bacterial colonization of epithelial cells. Epidemiological investigations are underway to assess the influence of RSV infection on carriage of meningococci or Hib.

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