MICROBIAL PATHOGENESIS

Bactericidal activity of a monocytic cell line (THP-1) against common respiratory tract bacterial pathogens is depressed after infection with respiratory syncytial virus

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Non-typable *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis* and respiratory syncytial virus (RSV) are commonly isolated from patients during the course of chronic obstructive pulmonary disease (COPD). Earlier studies found that virus infection enhanced binding of bacterial respiratory pathogens to epithelial cells *in vitro*. The objective of the present study was to assess the effect of RSV infection of a human monocytic cell line on bactericidal activity and cytokine production in response to these bacterial respiratory pathogens. The effect of RSV infection on binding, uptake and intracellular killing of bacteria by a human monocytic leukaemia cell line, THP-1, was assessed. Cell culture supernates were examined with a mouse fibroblast cell assay for tumour necrosis factor- α (TNF- α) bioactivity. Expression of CD14, CD11a, CD18, CD15 and CD29 on uninfected and RSV-infected THP-1 cells was assessed by flow cytometry in relation to differences in bacterial binding. RSV infection of THP-1 cells significantly decreased their ability to bind and kill bacteria. Compared with uninfected cells, fewer bacteria bound to RSV-infected THP-1 cells and the surface antigens that have been reported to bind bacteria were expressed at lower levels on RSV-infected cells. RSV-infected cells incubated with bacteria exhibited less TNF- α bioactivity than uninfected cell incubated with bacteria. The results elucidate some of the mechanisms involved in the increased susceptibility of virus-infected patients to secondary bacterial infection. Reduced bacterial killing by virus-infected monocytes might contribute to reduced clearance of bacteria from the respiratory tract and damage elicited by the bacteria or cytokine response in COPD patients.

Introduction

Chronic obstructive pulmonary disease (COPD), which includes chronic bronchitis and emphysema, is an important cause of ill health in older age groups. Whilst cigarette smoking is a major risk factor for the condition [1], the precise role of microbial infections in its development and progress has yet to be elucidated [2, 3].

Non-typable *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* are common bacterial species isolated from patients during episodes of exacerbation in the course of COPD and asthma [4–7]. Respiratory syncytial virus (RSV) is an

important viral pathogen in older groups, because complete immunity does not follow RSV disease and re-infections are not uncommon [8]. Fagon and Chastre [9] reviewed studies comparing the tracheo-bronchial microflora of patients during acute exacerbations and stable periods in the course of COPD; significant differences in isolation rates in these studies were found only for viruses (influenza virus and RSV). These studies did not measure the bacterial flora of the respiratory tract or the effect of acute viral infections on inflammation due to the resident flora. Infection of epithelial cells with RSV increased binding of respiratory bacterial pathogens [10–12]. Patients with RSV infections showed a significant rise of antibodies to *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* [13].

Viral infections might also alter the bactericidal

mechanisms in the respiratory tract. Alveolar macrophages play an important role in killing and inhibition of replication of inhaled micro-organisms and in inflammation in the respiratory tract due to these agents. A human monocytic leukaemia cell line, THP-1 [14], possesses the properties of alveolar macrophages [15] and was adopted as an appropriate model to study interaction between RSV and bacterial species isolated from patients with COPD.

The first objective of this study was to examine the effect of RSV infection of THP-1 cells on binding, ingestion and intracellular survival of strains of *H. influenzae*, *S. pneumoniae* and *M. catarrhalis*. Surface antigens on monocytes, CD14, CD11a, CD18, CD15 and CD29 – some of which act as bacterial receptors [16, 17] – can be modified during inflammation [18]. RSV infection suppressed the expression of LFA-1 (CD11a + CD18) on human monocytes [19]. The second objective of this study was to examine the changes in the cell-surface antigens associated with RSV infection in relation to the ability of monocytes to bind and ingest bacterial isolates. Tumour necrosis factor- α (TNF- α) plays a role in defence against viral [20, 21] and bacterial infection [22, 23], but its release also results in inflammation in the respiratory tract and contributes to the systemic symptoms in patients with COPD [24, 25]. The third objective was to examine the effect of RSV infection and different strains of bacteria, or both, on TNF- α release from THP-1 cells.

Materials and methods

RSV

The Edinburgh strain of RSV (subgroup A) was harvested from HEp-2 cells maintained in maintenance medium (MM) which consisted of RPMI-1640 supplemented with fetal calf serum (FCS) 1% and 2 mM L-glutamine, penicillin 100 IU/ml and streptomycin 200 μ g/ml. The suspension was tested by immunofluorescence for presence of mycoplasma. The concentration was adjusted to 2×10^6 plaque forming units/ml [10].

Bacteria

There were two isolates each of nontypable *H. influenzae* (HI1 and HI2), *M. catarrhalis* (MC1 and MC2) and *S. pneumoniae* (types 3 and 6) from patients with exacerbation of COPD obtained from the Clinical Bacteriology Laboratory of the department. *H. influenzae* and pneumococcal isolates were sensitive to ampicillin. *M. catarrhalis* strain MC1 grew on New York City medium with antibiotics selective for the pathogenic neisseria: lincomycin 1 μ g/ml, colistin 6 μ g/ml, amphotericin 1 μ g/ml and trimethoprim lactate 6.5 μ g/ml. *M. catarrhalis* MC2 did not grow on this medium. Overnight growths of *H. influenzae* and *M. catarrhalis* on boiled blood agar and *S.*

pneumoniae on blood agar were collected in phosphate-buffered saline (PBS) and washed twice by centrifugation at 2500 g for 10 min. Heavy bacterial suspensions in MM without antibiotics were stored in small volumes at -20°C for up to 3 months for use in the assays. Concentrations of live bacteria in the frozen samples were determined by plating triplicate samples (5 μ l) of appropriate dilutions in PBS on appropriate media for determination of colony forming units (cfu) after overnight growth in air with CO_2 5% at 37°C .

Ethidium bromide-labelling of bacteria

The bacterial suspensions were washed with PBS and fixed with buffered paraformaldehyde (Sigma) 1% for 30 min in a water bath at 37°C . The bacteria were washed twice with PBS and incubated with ethidium bromide (EB; Sigma) 50 μ g/ml for 20 min in a water bath at 37°C . The bacteria were washed twice and the total count was adjusted to 4×10^8 /ml in PBS by direct microscopy. The labelled bacteria were held in small volumes at -20°C for up to 3 months.

THP-1 cells

THP-1 cells (European Collection of Animal Cells Cultures, Salisbury, Wilts) were cultured in growth medium (GM) which contained the same components as MM except for the higher concentration of FCS (10%) and the presence of mercapto-ethanol (2×10^{-5} M). The suspension was tested as described earlier for presence of mycoplasma. The cells were kept at 37°C in CO_2 5%. Fresh cultures of cells were infected with RSV at a multiplicity of infection of 2. Overnight cultures of uninfected and RSV-infected cells were washed with MM without antibiotics by centrifugation at 300 g for 7 min and the counts were adjusted to 1×10^6 /ml in this medium by microscopy, for use in the experiments.

The proportions of monocytes infected with RSV in the samples were determined by flow cytometry by indirect immunofluorescence. The proportions were compared in one experiment with a mouse monoclonal antibody (MAb) to a viral surface glycoprotein G [10] or a convalescent serum from a patient with RSV infection previously absorbed with THP-1 cells and appropriately diluted in PBS. FITC-conjugated anti-mouse immunoglobulin or anti-human immunoglobulin antibodies (Sigma) were used to detect primary antibodies on cells. The convalescent serum was used to detect RSV-infected cells in subsequent experiments. The viability of RSV-infected and uninfected THP-1 cells was determined by trypan blue exclusion.

Bacterial binding

Uninfected and RSV-infected THP-1 cells (2×10^5) in capped plastic tubes were mixed with EB-labelled bacteria to provide a ratio of 10 bacteria per cell and

incubated at 37°C in an orbital incubator (40 rpm). Samples were removed after incubation for 0 or 30 min and washed three times with ice-cold PBS by centrifugation at 300 g for 7 min to remove unbound bacteria from the pellet. The cells were suspended in 1 ml of PBS and stored in the dark at 4°C until analysed within 24 h by flow cytometry.

Flow cytometric analysis

The main cell population was gated by forward and side light scatter to exclude debris and cell aggregates. The percentage of cells with fluorescence greater than the background was determined on a histogram produced by log-amplified red fluorescence signals and mean fluorescence of the positive population on a histogram produced by linear signals. The values were multiplied to obtain a binding index (B_{Ind}) for each sample.

Ingestion of bacteria

To measure the fluorescence from bacteria ingested by cells, fluorescence from extracellular bacteria was quenched in each sample (1 ml) with 20 μ l of crystal violet (BDH) 0.05% in 0.15 N sodium chloride. As the optical density of the soluble dye and its quenching effect increases with time, flow cytometry was performed when the OD₅₉₅ reading of the sample was 500–600. In this range >90% of the EB-labelled bacteria outside the cells were completely quenched. Crystal violet at higher OD values is membrane permeable and can quench intracellular bacteria. The laser power for these samples was adjusted to compensate for the decrease in background autofluorescence of the control samples due to quenching. An ingestion index (I_{Ind}) for each sample was calculated by multiplying the percentage of fluorescent cells and the mean fluorescence as described above.

Intracellular survival of bacteria

Uninfected and RSV-infected THP-1 cells were incubated with live unlabelled bacteria under the above conditions for 30 min. Extracellular bacteria were killed by adding gentamicin 30 μ g/ml and ampicillin 50 μ g/ml for 15 min at 37°C. After three washes with PBS, the cells were resuspended in 100 μ l of PBS and lysed with an equal volume of sodium lauryl sulphate 0.05% in sterile distilled water. Samples were immediately plated in triplicate for determination of cfu as described above. For a time course study of intracellular survival and growth of bacteria, cells were incubated for different periods before lysing and plating.

Binding of anti-CD antibodies to cells

The flow cytometry method described previously to detect host cell antigens on buccal epithelial cells was

used in these experiments [26]. Uninfected and RSV-infected THP-1 cells were incubated at 4°C for 30 min with the following mouse MAbs: CD11a (Dako) diluted 1 in 20, CD18 (Dako) diluted 1 in 20, CD15 (Scottish Antibody Production Unit, Carlisle) diluted 1 in 20, CD14 (Dako) diluted 1 in 10 and CD29 (Serotec) diluted 1 in 20. The samples were washed three times with PBS. Fluorescein isothiocyanate (FITC)-labelled anti-mouse immunoglobulin antibody (Sigma) diluted 1 in 100 was used to detect binding of the primary antibodies to cells. The samples were washed three times and fixed with buffered paraformaldehyde (Sigma) 0.5%. Cells with fluorescence greater than the control (cells treated only with the FITC-labelled second antibody) were assessed for mean fluorescence and the binding index was calculated as described above.

TNF- α bioactivity

Uninfected and RSV-infected THP-1 cells were incubated in CO₂ 5% at 37°C with unlabelled live or EB-labelled fixed bacteria at a ratio of 10 bacteria per cell in 24-well tissue culture plates (Costar). After incubation for 24 h, supernates from individual wells were collected for determination of TNF- α . A bioassay with L-929 cells (a mouse fibroblast cell line) was used to determine TNF- α activity [27].

Statistical analysis

Paired t tests were used to analyse data from binding and ingestion of bacteria, and for binding of anti-CD MAbs to uninfected or RSV-infected cells. Wilcoxon's test for matched pairs was applied to the data from experiments measuring intracellular survival of bacteria. The data for TNF- α production by uninfected or RSV-infected THP-1 cells under various conditions were also analysed by paired t tests.

Results

The proportions of RSV-infected cells were similar with both mouse anti-G MAb and absorbed human convalescent serum. At 24 h after infection, 40–50% of THP-1 cells were infected with RSV. RSV infection did not affect the viability of cells 24 h after infection.

Bacterial binding, ingestion and survival

Bacteria were able to bind to cells at 0 min (data not shown), but intracellular bacteria were not detected at this time either by quenching the external bacteria or by determination of intracellular survival. Data from nine experiments on binding of *H. influenzae*, *M. catarrhalis* and pneumococci are presented in Fig. 1 and on ingestion of these bacteria in Fig. 2.

H. influenzae. The binding observed for strain HI1 to

Fig. 1. Binding indices (SE) of *H. influenzae* isolates (HI1 and HI2), *M. catarrhalis* isolates (MC1 and MC2) and *S. pneumoniae* types 3 and type 6 (SP3 and SP6) to THP-1 and RSV-infected THP-1 cells (mean of nine experiments); ■, uninfected THP-1 cells; ▒, RSV-infected THP-1 cells.

uninfected THP-1 cells was 50% of that observed for strain HI2 ($p < 0.05$). Strain HI1 also bound significantly less to RSV-infected cells than strain HI2 ($p < 0.01$). Compared with uninfected cells, binding of both the strains to cells infected with RSV was significantly reduced: HI1, $p < 0.01$, 95% CI -5520 , -18141 ; HI2, $p < 0.02$, 95% CI -1588 , -25237 . RSV infection of cells reduced the ingestion of both strains, but this was significant only for HI1 ($p < 0.05$, 95% CI -791 , -12793). Both the isolates survived better in RSV-infected cells than in uninfected cells ($p < 0.02$, HI1, $Z = -2.366$; $p < 0.05$, HI2, $Z = -2.1974$) (Fig. 3).

M. catarrhalis. Compared with strain MC2, strain MC1 bound in greater numbers to uninfected THP-1 cells ($p < 0.001$) and to RSV-infected cells ($p < 0.005$).

Both strains MC1 and MC2 bound significantly less to RSV-infected cells than to uninfected cells: MC1, $p < 0.01$, 95% CI -6796 , -11840 ; MC2, $p < 0.002$, 95% CI -12258 , -28233 . RSV infection of cells reduced the ingestion of both strains (MC1, NS; MC2, $p < 0.05$, 95% CI -335 , -31328). Neither of the isolates survived in uninfected or RSV-infected THP-1 cells in the conditions used in the study.

S. pneumoniae. Compared with strain SP6, strain SP3 bound significantly more to uninfected cells ($p < 0.05$) and to RSV-infected cells ($p < 0.05$). Compared with uninfected cells, lower numbers of strain SP3 bound to RSV-infected cells ($p = 0.051$, 95% CI 55 , -23737). There was no significant difference in binding of strain SP6 to uninfected or RSV-infected cells. A decrease in ingestion of strain SP3 by RSV-infected cells was observed ($p = 0.059$, 95% CI 1212 , -45717). There was no significant difference for strain SP6 in ingestion by uninfected or RSV-infected THP-1 cells. Significantly greater numbers of strain SP3 survived in RSV-infected cells than in uninfected cells ($p < 0.05$, $Z = -2.0226$) (Fig. 3). Strain SP6 showed evidence of only occasional survival in uninfected cells, which was twice as high in RSV-infected cells.

Effect of RSV infection on binding of anti-CD MABs to cells

Mean B_{ind} for the MABs directed towards the cell surface antigens of THP-1 cells are summarised in Fig. 4a and b. In seven experiments, RSV infection resulted in significant decreases in binding of the following MABs: anti-CD11a by 35% ($p < 0.05$, 95% CI -20 , -50); anti-CD18 by 24% ($p < 0.001$, 95% CI -16 , -32); anti-CD14 by 28% ($p < 0.05$, 95% CI -3 , -53) and anti-CD15 by 30% ($p < 0.05$, 95% CI -3 , -57). There was an increase of 9% in binding of anti-CD29 to RSV-infected THP-1 cells, but it was not statistically significant.

Fig. 2. Ingestion indices (SE) of *H. influenzae* isolates (HI1 and HI2), *M. catarrhalis* isolates (MC1 and MC2) and *S. pneumoniae* types 3 and type 6 (SP3 and SP6) to THP-1 and RSV-infected THP-1 cells (mean of nine experiments); ■, uninfected THP-1 cells; ▒, RSV-infected THP-1 cells.

Fig. 3. Survival of *H. influenzae* isolates (HI1 and HI2) and *S. pneumoniae* type 3 (SP3) in uninfected and RSV-infected THP-1 cells (mean of seven experiments); ■, uninfected THP-1 cells; ▒ RSV-infected THP-1 cells.

Effect of RSV infection and bacteria on TNF- α bioactivity of cells

TNF- α bioactivity in supernates of uninfected or RSV-infected THP-1 cells incubated with either live unlabelled or fixed EB-labelled bacteria was determined (Fig. 5). In seven experiments, compared with THP-1 cells to which no bacteria were added, the bioactivity was increased by incubation of THP-1 cells with live strains: HI1, $p < 0.01$ (95% CI 11.5, 45.9);

HI2, $p < 0.001$ (95% CI 24.3, 50.6); MC1, $p < 0.05$ (95% CI 8.5, 59.2); MC2, $p < 0.05$ (95% CI 5.1, 47.2); SP3, $p < 0.001$ (95% CI 48.6, 65.9) or SP6, $p < 0.005$ (95% CI 15.5, 44.5). Differences in the levels of TNF- α induced were not statistically significant between strains of the same species.

Compared with THP-1 cells to which no bacteria were added, the TNF- α bioactivity was increased by incubation of THP-1 cells with EB-labelled fixed strains: HI1, $p < 0.05$ (95% CI 4.1, 31.9); HI2, $p < 0.001$ (95% CI 19, 42.4); MC1, $p < 0.002$ (95% CI 23.1, 63.2); MC2, $p < 0.001$ (95% CI 38.9, 59); SP3, $p < 0.02$ (95% CI 5.9, 29.5) or SP6, NS. Compared with fixed strain HI1, fixed strain HI2 elicited significantly more TNF- α bioactivity ($p < 0.005$, 95% CI 6, 19.3). Fixed MC2 and SP3 strains elicited greater TNF- α bioactivity compared with fixed MC1 and SP6 strains, respectively, but the differences were not statistically significant.

RSV infection increased TNF- α bioactivity from THP-1 cells incubated with live HI1, HI2 or MC2 strains, but the differences were not statistically significant. Compared with uninfected cells, TNF- α bioactivity was decreased for RSV-infected cells incubated with live strain MC1, $p < 0.002$ (95% CI -49.3, -18.4); SP3, $p < 0.001$ (95% CI -47.6, -20.4) or SP6, $p < 0.005$ (95% CI -23.2, -8.2).

RSV infection increased TNF- α bioactivity from THP-1 cells incubated with fixed HI1 or HI2 strains, but the differences were not statistically significant. Compared with uninfected cells, there was decreased TNF- α

Fig. 4. (a) Binding of MAb to CD11a, CD18, CD14 and CD29 to uninfected (■) and RSV-infected (▒) THP-1 cells (mean of seven experiments). (b) Binding of MAb to CD15 to uninfected (■) and RSV-infected (▒) THP-1 cells (mean of seven experiments).

Fig. 5. Percentage cytotoxicity for L-929 cells caused by TNF- α in supernates from uninfected or RSV-infected THP-1 cells incubated with different isolates of live (□) or fixed (■) bacteria obtained from seven experiments. HI, *H. influenzae*; MC, *M. catarrhalis*; SP, *S. pneumoniae*.

bioactivity with RSV-infected cells incubated with fixed MC1, MC2, SP3 or SP6 strains, but the results were significant only for strain SP3 ($p < 0.01$, 95% CI -25.5 , -5.2).

Discussion

Although no significant differences in rates of isolation of bacterial species during exacerbation and stable periods were found in patients with COPD [9], the density of bacterial colonisation of the respiratory tract might contribute to exacerbation. Secondary bacterial infections following virus infections are thought to be associated with enhanced bacterial binding, suppression of host immune responses and bactericidal functions associated with viral infections [28].

Previous studies have shown that RSV infection increases bacterial binding to epithelial cells [10–12, 26]. When infected with RSV, murine alveolar macrophages showed increased cytokine and decreased bactericidal functions [29]. As monocytes play an important role in bactericidal activity in the respiratory tract, the present study used THP-1 cells as a model to examine the effect of RSV infection on binding, ingestion and killing of bacteria.

The bronchial tree has been envisaged as a relatively sterile organ in healthy individuals, in which continuous exposure to inhaled micro-organisms is met with appropriate host defence mechanisms. Heavier growths of bacteria over prolonged periods in the respiratory tract in COPD as a result of inefficient bactericidal mechanisms might cause further damage to the tract due to release of inflammatory cytokines. With this in mind, this study examined the levels of TNF- α , a bactericidal agent [22] and a very potent pro-

inflammatory cytokine, from THP-1 cells in response to common microbial pathogens.

The first objective was to compare the differences in binding of individual isolates of each bacterial species and their killing by uninfected and RSV-infected THP-1 cells. RSV-infected cells demonstrated reduced levels of binding, ingestion and killing of most of the bacteria. The second objective was to assess the effects of RSV infection on surface antigens of the phagocytes that act as receptors for bacteria. Decreased ingestion of most of the bacteria by THP-1 cells infected with RSV might be due to the lower levels of initial bacterial binding observed with these cells. CD14, CD11a, CD18 and CD15 have been identified as receptors for several bacterial species [16, 17]. Lower levels of these antigens associated with RSV infection might contribute to the decreased bacterial binding observed in this study. However, a correlation test was not performed for BIs of the antibody and bacterial binding to cells, because the two types of experiments were performed on different preparations of cells. Previous studies have demonstrated a significant relationship between bacterial binding and expression of blood group antigens that act as receptors on epithelial cells [26, 30].

Increased TNF- α bioactivity contributes to inflammation, whereas a marked decrease can jeopardise mucosal protection against bacteria and bactericidal activity [31, 22]. The third objective of the present study was to compare TNF- α produced by uninfected and RSV-infected THP-1 cells in response to bacteria. Compared with cells not exposed to bacteria, various degrees of increased responses were observed with all the bacterial isolates. RSV infection reduced TNF- α bioactivity from cells incubated with strains MC1, SP3 and SP6. RSV infection in human alveolar macrophages can persist for at least 25 days after infection in vitro [32] and in a significant number of patients with COPD [33]. Persistently lower TNF- α responses to the bacteria observed with RSV infection combined with decreased intracellular bactericidal activity might prolong bacterial disease in patients with COPD and cause chronic damage to the respiratory tract.

In conclusion, the differences observed between uninfected and RSV-infected THP-1 cells in the pattern of binding, intracellular killing of bacteria and TNF- α production in response to bacteria may explain the increased susceptibility of virus-infected patients to secondary bacterial infections. Bacteria that escape virus-infected monocytes might have a greater opportunity to grow in the milieu of the respiratory tract and cause disease. Further work to examine the effects of specific antibodies to *S. pneumoniae* on intracellular bacterial survival and production of cytokines from monocytes is under way.

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