Restricted replication of respiratory syncytial virus in human alveolar macrophages

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The cellular factors that regulate infection and replication of respiratory syncytial virus (RSV) in human alveolar macrophages were examined. RSV-exposed alveolar macrophages demonstrated a time-dependent expression of viral glycoproteins, maximal by 24 h post-infection resulting in infection of approx. 38% of the cells. Essentially all (33%) of these freshly isolated alveolar macrophages replicated RSV as shown by infectious centre assays. This RSV-permissive subpopulation of alveolar macrophages consisted primarily of major histocompatibility class II-expressing cells as determined by fluorescence-activated cell sorting. Re-infection of alveolar macrophages did not significantly alter the number of cells infected or capable of replicating RSV. However, in vitro differentiation of alveolar macrophages prior to infection resulted in a significant (P < 0.05), time-dependent decrease (approx. sevenfold) in the number of cells that replicated virus. The mechanism by which cellular differentiation restricted RSV replication is unknown. Production of defective interfering particles did not account for this decrease. Alveolar macrophages infected with RSV produce a variety of cytokines potentially contributing to this restricted viral replication. Pretreatment with several of these cytokines did not affect viral infection or replication. However, tumour necrosis factor (TNFα) significantly (P < 0.05) decreased viral replication but only by 30 to 60%. Thus RSV replication is reduced by in vitro differentiation of alveolar macrophages and, to a lesser degree, by pretreatment with TNF.

Introduction

Respiratory syncytial virus (RSV) is the major cause of bronchiolitis in infants (Stott & Taylor, 1985; McIntosh & Chanock, 1990). The pathogenesis of RSV-induced bronchiolitis is poorly understood and no effective vaccine is currently available (Pringle, 1987; Kimman & Westenbrink, 1990). RSV causes injury to terminal bronchioles and alveoli accompanied by lymphocytic and monocytic cell infiltrates (Aherne et al., 1970; Neilson & Yunis, 1990). The mechanisms of injury of the human lung are thought to be secondary to viral replication in airway cells as well as the local immune response (Pringle, 1987; McIntosh & Chanock, 1990; Kimman & Westenbrink, 1990). This hypothesis is supported by evidence that human lung epithelial cells and alveolar macrophages, the major cell types present at the site of injury, are permissive to RSV infection in vivo (Neilson & Yunis, 1990; Panuska et al., 1992) and replicate virus efficiently in vitro (Panuska et al., 1990a; Becker et al., 1992).

Both in vivo and in vitro studies of lung epithelial cells demonstrate c.p.e., fusion resulting in syncytia, and cytolysis (Parry et al., 1979; Becker et al., 1992; Merolla et al., 1992). Recent in vivo studies indicate that major histocompatibility complex (MHC) class II (HLA-DR)-expressing alveolar macrophages are also targets for RSV infection (Panuska et al., 1992). In vitro studies indicate that RSV initially replicates efficiently in alveolar macrophages but this progressively decreases with increasing time of culture varying by approx. 50-fold by 15 days post-infection (p.i.) (Panuska et al., 1990a). Furthermore, productively infected alveolar macrophages do not demonstrate cytolysis, loss of viability, or syncytia up to 25 days p.i. (Panuska et al., 1990a). Finally, only approx. 35% of these cells are permissive to RSV infection (Midulla et al., 1989) but it is not known whether all infected cells can replicate RSV. The mechanism(s) of this restricted infection of alveolar macrophages and progressive decrease in RSV repli-
cation has not been defined. Because RSV replication in the normal infant is limited and virus shedding generally lasts less than 2 weeks (McIntosh & Chanock, 1990), the factors that limit RSV replication in differentiated human lung cells may provide insight into pathogenesis as well as local mechanisms that restrict viral replication.

The alveolar macrophage has a major role in defending the lower respiratory tract against invading pathogens including viruses (Morahan et al., 1985). Paradoxically, alveolar macrophages replicate RSV (Panuska et al., 1990a; Becker et al., 1992), other paramyxoviruses (Mills, 1979), orthomyxoviruses (Nain et al., 1990), coronavirus (Laude et al., 1984), retroviruses (Gendelman et al., 1985; Chayt et al., 1986) and DNA viruses (Rose et al., 1986; Nash et al., 1988) which should increase viral burden. However, most studies have shown that only a fraction of the alveolar macrophage population is permissive to viral infection and/or replication and, with RSV, viral replication is less efficient than that seen with epithelial cells (Becker et al., 1992). Thus alveolar macrophages are composed of RSV-resistant and -permissive cell populations and additional undefined factors regulate RSV infection and replication in these populations.

Alveolar macrophages consist of subpopulations of cells differing in cytokine production (Elias et al., 1985), metabolism of arachidonic acid (Chandler & Fulmer, 1987), initiation of lung immune responses (Ferro et al., 1987) and expression of MHC class II molecules (Nicol et al., 1989). MHC class II expression varies with the state of differentiation of macrophages and can alter virus–receptor binding (Inada & Mims, 1984; Mann et al., 1988) but whether differentiation alters RSV interaction with alveolar macrophages has not been studied. In addition, RSV-infected alveolar macrophages produce potent cytokines with potential antiviral activities including interleukin 1 (IL-1) (Panuska et al., 1992), tumour necrosis factor (TNF) (Panuska et al., 1990b; Becker et al., 1991), interleukin 6 (IL-6) and interleukin 8 (IL-8) (Becker et al., 1991). Macrophages derived by in vitro differentiation then infected with RSV demonstrate increased production of IL-1 (Roberts et al., 1986; Salkind et al., 1991), prostaglandin E2 (PGE2) (Panuska et al., 1990b) and platelet-activating factor (PAF) (Villani et al., 1991). These cytokines and mediators, following binding to their cognate receptors, affect diverse biological processes modulating gene transcription thus altering immune responsiveness, as well as cellular maturation and differentiation (Nathan, 1987; Dinarello, 1989; Braquet et al., 1989), processes known to alter cellular interactions with virus. Macrophages can also restrict viral replication by inactivation of extracellular virus, lysis of virus-infected cells, secretion of enzymes competing for virus-dependent substrates, and production of defective interfering virus (for a review see Morahan et al., 1985). We examined each of these mechanisms to define the nature of restricted RSV replication in alveolar macrophages. Definition of the factors that regulate RSV replication in human lung cells may provide insight into strategies for controlling RSV replication in the human lung.

Methods

Broncho-alveolar lavage and preparation of cells. Following informed consent and approval of these studies by the Institutional Review Board of University Hospitals (Cleveland, Ohio, U.S.A.), 23 non-smoking healthy adults without a history of a respiratory tract infection within the preceding 4 weeks underwent bronchoscopy and broncho-alveolar lavage (BAL) by methods we have previously described (Panuska et al., 1990a). BAL cells (106 cells/ml) were allowed to adhere for 1 h to tissue culture dishes and non-adherent cells were removed by washing with RPMI 1640, 10% (v/v) fetal bovine serum (FBS) (Hyclone), 2 mM-L-glutamine, 1 mM-non-essential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin and 0·25 μg/ml of amphotericin B (culture medium) (all supplements from Sigma). CVI cells were washed with Earle’s balanced salt solution (JRH Biosciences). 1% (v/v) FBS, and inoculated with RSV at 0·1 p.f.u./cell. Virus was allowed to adsorb for 2 h at 37 °C in 5% CO2. The cell monolayers were then washed with MEM and cultured for 48 h at 37 °C in 5% CO2 with MEM containing 3% (v/v) FBS with the supplements listed above. The cells and medium were made 30 mM-MgSO4, 16 mM-HEPES, 5 mM-NaCl, harvested by scraping, sonicated for 60 s twice at the maximum output from a Fischer Sonic Dismembrator Model 300 (Fischer Scientific) and stored at −70 °C.

Alveolar macrophages were cultured at 106 cells/ml at 37 °C in 5% CO2 in culture medium for the times indicated prior to infection with RSV or u.v.-light-inactivated RSV (u.v.RSV) (10 cm, 15 W for 1 h). Cells were exposed to RSV or u.v.RSV for 2 h at 37 °C in 5% CO2 at the m.o.i. given. After 2 h, viral inoculum was removed, adherent cells were washed four times and incubated under the above conditions for the times shown. Acidine orange and ethidium bromide staining of adherent cultures was used as previously described (Panuska et al., 1990a) to assess the viability of uninfected or infected alveolar macrophage cultures. The viability was greater than 90% at all time-points and did not differ between uninfected and infected cultures.

Assays for viral infection. Alveolar macrophage cultures exposed to RSV were assessed for viral infection by immunofluorescence microscopy as previously described (Midulla et al., 1989). Briefly, alveolar macrophage cultures were harvested using a rubber policeman and the cells were resuspended at 8×104 cells/ml in ice-cold Hanks’ balanced salt solution. Cells were then fixed with ice-cold methanol/acetone (1:1 v/v) for 10 min, followed by incubation for 30 min at 37 °C with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (MABs) directed against RSV surface glycoproteins (a kind gift from Bartels Diagnostics). Cells were washed three times with PBS and assessed by phase contrast and epifluorescence microscopy. A minimum of 200 cells were enumerated and the percentage of infected cells was
were then overlaid with 0.5% (w/v) agarose in a final concentration of 1%. Viral titre was determined from sonicated alveolar macrophage lysates by methods we have previously described (Panuska et al., 1990a).

Pre-treatment of cells prior to RSV infection. Alveolar macrophages were treated with the following cytokines and mediators at the concentrations listed in Table 3 for 16 h prior to infection with RSV at an m.o.i. of 3. Cytokines and their sources were as follows: rTNFα (Genentech), IL-1β (Collaborative Research), rIL-6 was a kind gift of Dr D. Samols (Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio, U.S.A.), and rIL-8 was a kind gift of Dr J. Rankin (Pulmonary Division, Department of Medicine, Yale University, New Haven, Conn., U.S.A.). Cells were also treated with PGE_{2} (Cayman Chemical Corporation), PAF and lys-PAF (Calbiochem). After infection with RSV for 24 h, cultures were harvested and assessed by immunofluorescence and ICA as described above.

MAbs to HLA-DR framework determinants included L203, L227 and L243 while isotype control MAbs included HB4 and HB121 (all from ATCC). The dilutions of L203, L227 and L243 that maximally detected HLA-DR-positive alveolar macrophages were determined by twofold serially diluting each MAb and enumerating positive cells by indirect immunofluorescence. Parallel samples of alveolar macrophages were reacted with a PE-conjugated MAb to HLA-DR (Becton-Dickinson) to determine the maximum number of HLA-DR-positive cells. To ensure saturation of HLA-DR framework determinants, L203, L227 and L243 were used at fourfold higher concentrations than necessary to stain all HLA-DR-positive cells.

Statistics. Results are presented as mean ± SEM or ± SD as indicated in the text; probabilities (P) were determined by paired t-tests and were considered significant at < 0.05.

Results

RSV infection and HLA-DR expression by alveolar macrophages

RSV infection of alveolar macrophages was initially assessed by direct immunofluorescence. Alveolar macrophages demonstrated a time-dependent expression of viral proteins first detected at 2 h post-viral addition (0 h p.i.) (3 p.f.u./cell) yielding 6.1 ± 1.1% infected cells which became maximal by 12 to 96 h p.i. (38 ± 3% infected cells) (Fig. 1a). At 2 to 6 h p.i., the rare infected cells demonstrated a few membrane-associated fluorescent granules (Fig. 1b) that by 12 h p.i. started to become diffuse within the cytoplasm (ii) and appeared maximal by 24 h p.i. (iii). By 96 h p.i., alveolar macrophages had markedly enlarged but continued to express viral proteins (iv). Alveolar macrophages exposed to u.v.-light-inactivated RSV (non-infectious, same dose) did not express viral proteins at any of these time points (not shown), suggesting that u.v. treatment may interfere with viral adsorption. Therefore, infectious RSV was absolutely required for viral protein expression.

These results suggested that alveolar macrophages were composed of subpopulations of cells resistant or permissive to RSV infection consistent with previous results (Panuska et al., 1990a). To characterize the virus-permissive cell population further, alveolar macrophages 24 h after RSV or u.v.-RSV exposure were analysed for RSV and HLA-DR protein expression by two-colour FACS analyses. Fig. 2 shows a representative experiment from a single donor. Alveolar macrophages showed comparable levels of green (vertical) and red (horizontal) auto-fluorescence (a, e) in the absence of MAbs to RSV and HLA-DR, respectively. The majority of both u.v.-RSV- and RSV-exposed cells expressed HLA-DR molecules (direct immunofluorescence with a PE-conjugated MAb) (b, f). As expected, u.v.-RSV-exposed alveolar macrophages failed to express RSV proteins (c) (direct immunofluorescence with FITC-conjugated anti-RSV MAbs). However, a subpopulation of alveolar macrophages exposed to infectious RSV intensely stained for RSV proteins (g). In addition, alveolar macrophages exposed to infectious RSV demonstrated a discrete population of cells expressing both RSV proteins and HLA-DR molecules (h) not seen with u.v.-RSV-exposed controls (d).

Co-expression of RSV proteins and HLA-DR molecules by alveolar macrophages were confirmed in six additional donors and combined results from these donors are shown in Table 1. In the absence of the MAbs
to RSV and HLA-DR, virtually all alveolar macrophages were unstained (Table 1). The majority of alveolar macrophages were HLA-DR-positive and demonstrated a significant ($P < 0.01$) increase in their mean log fluorescent intensity (MLFI) values shown in Table 1) compared to unstained or isotype-stained controls (see Fig. 1).

(a)

(b)

Fig. 1. (a) Kinetics of RSV infection of alveolar macrophages. Alveolar macrophages ($10^6$ cells/ml) were exposed for 2 h to RSV at an m.o.i. of 3. Virus was removed and cultures were washed extensively to remove unadsorbed virus. The cells were harvested by scraping at the times indicated, fixed with ice-cold methanol-acetone, reacted with MAbs to RSV glycoproteins and the percentage of fluorescent cells was determined by epifluorescence and light microscopy. Results shown are mean ± s.i.m. (b) Kinetics of RSV protein expression by infected alveolar macrophages. Direct immunofluorescence detection of RSV glycoproteins was determined at 2 h (i), 12 h (ii), 24 h (iii) and 96 h (iv) p.i. Bar marker represents 25 μm.
RSV replication in alveolar macrophages

Fig. 2. RSV and HLA-DR protein expression by alveolar macrophages. Alveolar macrophages (10⁶ cells/ml) from a single representative donor were exposed to u.v. RSV or RSV at an m.o.i. of 3 as indicated, harvested after 24 h, and fixed with ice-cold methanol. Cells were untreated or treated with MAbs to RSV conjugated to FITC and/or a MAb to HLA-DR conjugated to PE, washed, and analysed by FACS. A total of 20000 cells per condition were analysed. Relative fluorescence intensity for RSV proteins (FITC MAbs), vertical axis, and HLA-DR proteins (PE MAb), horizontal axis, are displayed.

Table 1. FACS analysis of RSV-exposed human alveolar macrophages*

<table>
<thead>
<tr>
<th>Condition</th>
<th>MAb to RSV (FITC)</th>
<th>MAb to HLA-DR (PE)</th>
<th>Unstained cells</th>
<th>RSV + (FITC)</th>
<th>HLA-DR + (PE)</th>
<th>RSV+ and HLA-DR +</th>
</tr>
</thead>
<tbody>
<tr>
<td>−RSV</td>
<td>−</td>
<td>−</td>
<td>99 ± 0.7</td>
<td>0.2 ± 0.4 (331 ± 43)</td>
<td>0.2 ± 0.2 (261 ± 129)</td>
<td>0.5 ± 0.5</td>
</tr>
<tr>
<td>+RSV</td>
<td>−</td>
<td>−</td>
<td>99 ± 0.5</td>
<td>0.1 ± 0.1 (336 ± 58)</td>
<td>0.2 ± 0.4 (283 ± 76)</td>
<td>0.3 ± 0.5</td>
</tr>
<tr>
<td>−RSV</td>
<td>−</td>
<td>+</td>
<td>32 ± 8</td>
<td>0.1 ± 0.1 (539 ± 52)</td>
<td>63 ± 10 (541 ± 48)</td>
<td>4 ± 4</td>
</tr>
<tr>
<td>+RSV</td>
<td>−</td>
<td>+</td>
<td>48 ± 17</td>
<td>0.2 ± 0.3 (367 ± 20)</td>
<td>43 ± 10 (509 ± 71)</td>
<td>7 ± 10</td>
</tr>
<tr>
<td>−RSV</td>
<td>+</td>
<td>−</td>
<td>94 ± 6</td>
<td>0.4 ± 0.4 (342 ± 41)</td>
<td>0.3 ± 0.4 (302 ± 28)</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>+RSV</td>
<td>+</td>
<td>−</td>
<td>56 ± 8</td>
<td>0.3 ± 0.3 (515 ± 52)</td>
<td>1.4 ± 3 (375 ± 89)</td>
<td>3 ± 4</td>
</tr>
<tr>
<td>−RSV</td>
<td>+</td>
<td>+</td>
<td>31 ± 12</td>
<td>0.4 ± 0.5 (403 ± 63)</td>
<td>65 ± 15 (563 ± 77)</td>
<td>4 ± 5</td>
</tr>
<tr>
<td>+RSV</td>
<td>+</td>
<td>+</td>
<td>36 ± 13</td>
<td>0.6 ± 0.6 (536 ± 40)</td>
<td>16 ± 10 (566 ± 38)</td>
<td>34 ± 12</td>
</tr>
</tbody>
</table>

* Results represent mean ± s.e.m. for six separate donors for the percentage immunofluorescence-positive cells; the number in parentheses represent mean ± s.e.m. for the MLFI.

Methods). The FACS parameters used to determine the percentage of HLA-DR-positive cells were designed to discriminate accurately between auto- and PE-fluorescence. RSV infection resulted in a small but significant (P < 0.05) decrease in the size of this HLA-DR-positive population without significantly altering the MLFI. Thus RSV infection increased the amount (density) of HLA-DR molecules on each alveolar macrophage. Mock-infected alveolar macrophages did not react with the MAbs to RSV whereas infectious virus caused a significant (P < 0.01) increase in both the number of FITC-positive cells and their MLFI. Alveolar macrophages treated with MAbs to RSV and HLA-DR and mock-infected demonstrated HLA-DR staining equivalent to cells reacted with anti-HLA-DR alone. Infectious virus yielded a discrete population of cells that expressed both RSV and HLA-DR molecules accounting for nearly all infected cells. Thus alveolar macrophages permissive to RSV infection consisted primarily of the HLA-DR-positive subpopulation.

Next it was assessed whether HLA-DR molecules were directly participating in RSV infection of alveolar macrophages. A panel of MAbs to HLA-DR framework determinants alone, or combined, at saturating concentrations (see Methods) were reacted with alveolar macrophages for 1 h prior to virus exposure. RSV was allowed to adsorb for 1 h and removed by washing. The number of infected cells was then determined by fluorescence microscopy at 24 h.p.i. Cells pretreated with anti-HLA-DR antibodies demonstrated 31 ± 9% infected cells compared to 33 ± 11% in cells reacted with isotype control antibodies at equivalent concentrations.
Table 2. Effects of single infection or reinfection on the number of alveolar macrophages infected or replicating RSV

<table>
<thead>
<tr>
<th>Assay</th>
<th>Alveolar macrophages</th>
<th>CV1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single infection</td>
<td>Reinfection (4 × dose)</td>
</tr>
<tr>
<td>Immunofluorescence infection (%)</td>
<td>39 ± 8 ± 42</td>
<td>45 ± 8 ± 22</td>
</tr>
<tr>
<td>Infectious centre percentage replicating</td>
<td>56 ± 16</td>
<td>52 ± 12</td>
</tr>
</tbody>
</table>

* Results represent the mean ± s.e.m. for five separate donors.

\( n = 4, \ P = \text{not significant} \). These results suggested that blocking HLA-DR framework determinants did not prevent RSV infection of alveolar macrophages.

**Correlation of RSV infection with virus replication in alveolar macrophages**

The above results indicated that alveolar macrophage cultures contained a subpopulation of cells permissive to RSV infection. RSV infection was productive in this cellular subpopulation. Cellular lysates from \( 10^6 \) alveolar macrophages at 2 h.p.i. contained less than 1000 p.f.u. but this increased significantly \( (P < 0.01) \) to \( 34000 ± 1800 \) p.f.u. by 24 h.p.i. \( (n = 4) \). Therefore, to quantify directly the number of cells replicating RSV, and to determine whether reinfection or increased viral dose altered the number of cells which replicated virus, ICA were performed. Infected alveolar macrophages (3 p.f.u./cell) from five donors were cultured for 24 h then mock-infected or reinjected with equal or fourfold higher doses of RSV and the percentage of infected cells that replicated virus was determined at 24 h after the final infection. Table 2 summarizes these data. Cells infected once, twice or reinjected with fourfold higher doses of virus showed no significant differences in either the number of infected cells (direct immunofluorescent staining for RSV proteins) or the number of cells that replicated RSV (ICA). Furthermore, the number of infected alveolar macrophages exceeded the number capable of replicating RSV under all conditions. In contrast, the number of RSV-infected CV1 cells was essentially identical to the number that replicated the virus (Table 2). Thus, infection and replication of RSV occurred in a discrete subpopulation of alveolar macrophages which appeared stable and regulated by cellular factors and not simply by viral dose.

**Effects of in vitro differentiation of alveolar macrophages on RSV infection and replication**

To assess directly whether the subpopulation of alveolar macrophages capable of replicating RSV was stable, the kinetics of RSV replication by alveolar macrophages cultured in vitro prior to infection was determined. Freshly isolated alveolar macrophages from five donors were either immediately exposed to RSV (2 h, 3 p.f.u./cell) or exposed to this viral dose after in vitro culture for 24 h and 48 h. The percentage of cells that replicated virus was determined at 0, 24 and 48 h.p.i. (Fig. 3). At 0 h.p.i. 64 ± 22% of freshly isolated alveolar macrophages replicated RSV; this was significantly higher \( (P < 0.05) \) than that found with cells cultured in vitro for 24 or 48 h prior to infection where 8.3 ± 32% and 7.1 ± 1% replicated virus, respectively. Similarly, at both 24 and 48 h.p.i.
freshly isolated alveolar macrophages replicated RSV more efficiently than did cells cultured for 24 or 48 h prior to infection (Fig. 3). The increased number of cells that replicated RSV at 0 h p.i. did not simply represent non-specific adsorption of virus because sonicated cellular lysates, at this time point, contained less than 1000 p.f.u./10^6 alveolar macrophages which would yield a maximum of 0.001 replicating cells by ICA. Sonication of alveolar macrophages would have to reduce the cell-associated virus titre by 34-fold to account for these results, an effect of sonication which we have not observed during preparation of viral cultures. Therefore, increasing time of in vitro culture decreased the number of cells that replicated virus. In contrast, the number of cells permissive to RSV infection determined in parallel samples of these cells did not differ (Fig. 3) indicating that in vitro differentiation selectively affected viral replication.

Clearly, in vitro induced differentiation of alveolar macrophages significantly reduced their capacity to replicate RSV. This restricted cellular replication of RSV reflected endogenous cellular mechanisms, but the possibility that production of defective interfering particles, or elaboration of cellular mediators that either directly (inactivation of virus) or indirectly (altering the state of differentiation of alveolar macrophages) contributed to decreased viral replication could not be excluded. These possibilities were examined sequentially.

Effects of macrophage supernatants on RSV titre

RSV-infected alveolar macrophage supernatants were assessed to see whether they were capable of inhibiting RSV replication. Cell-free supernatants from infected alveolar macrophages (3 p.f.u./cell, 10^6 cells/ml) from five donors harvested 24 h p.i. were mixed with stock RSV (10^5 p.f.u./ml) at the dilutions indicated in Fig. 4. These supernatants did not significantly alter the titre of stock RSV determined on CV1 cells at any of these dilutions. Similarly, alveolar macrophage supernatants harvested at 96 h p.i. and mixed with stock RSV yielded titres that agreed within ±10% of the expected titre (n = 2). Therefore, the production of defective interfering particles or viral inhibitory activities did not restrict virus infection and syncytium formation by CV1 cells.

Effects of alveolar macrophage cytokines/mediators on RSV infection and transmission

Although RSV-infected alveolar macrophage supernatants did not restrict virus infection of CV1 cells, it was next examined whether several cytokines/mediators, likely to be present in these supernatants, altered alveolar macrophage permissiveness to RSV. It was possible that these supernatants might interfere with virus production by altering the state of differentiation of alveolar macrophages through autocrine or paracrine mechanisms.

Alveolar macrophages (10^6 cells/ml) were exposed for 16 h to media controls, TNFα, IL-1β, IL-6, IL-8, PGE_{2}, PAF and lyso-PAF at the concentrations shown in Table 3. Cells were then exposed to RSV (3 p.f.u./cell), washed four times and incubated in the absence of these molecules for an additional 24 h. Both the number of infected cells and those which replicated RSV were determined. As shown in Table 3, pretreatment of alveolar macrophages with these molecules had no
significant effects on the number of infected cells. However, TNFα at 1 ng/ml significantly (P < 0.05) reduced the number of cells that replicated RSV whereas all other mediators showed no significant effects. Pre-treatment of alveolar macrophages with higher doses of IL-1β, IL-6 and IL-8 (100 ng/ml) yielded replication in 4.8 ± 1.3, 5.3 ± 0.6 and 5.7 ± 1.2% of the cells, respectively (n = 3) which did not differ from controls.

To exclude carry-over effects of TNFα on the ICA, CV1 cells were exposed to TNFα (1 to 100 ng/ml) at the time of addition of stock RSV. The number of viral plaques after 5 days did not differ between TNFα-exposed CV1 cells and media controls indicating that CV1 cells do not respond to the antiviral effect of TNFα. The effects of TNFα dose on the number of alveolar macrophages that replicated RSV were examined in three donors and representative results from a single donor (mean ± s.d. of triplicate samples) are shown in Fig. 5. Alveolar macrophages pretreated with TNFα at 0.01 ng/ml demonstrated minor effects on RSV replication (inhibition of viral replication, range 0 to 22%, n = 3) whereas alveolar macrophages pretreated with TNFα at 1 and 100 ng/ml demonstrated a decrease in the number of cells that replicated RSV; and TNFα which is rapidly induced in RSV-infected alveolar macrophages resulted in a progressive decrease in the number of cells that replicated virus; and TNFα which remains constant up to 96 h.p.i.; HLA-DR-positive alveolar macrophages contained a discrete RSV-permissive subpopulation of cells; only a fraction of the infected cells replicated RSV; in vitro culture of alveolar macrophages resulted in a progressive decrease in the number of cells that replicated virus; and TNFα which is rapidly induced in RSV-infected alveolar macrophages (Panuska et al., 1990b; Becker et al., 1991) restricted viral replication without altering the number of infected cells when administered prior to infection.

At 2 h.p.i. viral proteins were visualized as small membrane-associated granules in rare cells probably reflecting fusion of the virions with cell membranes and not viral protein synthesis because viral mRNA is not detectable this early (Panuska et al., 1990b). By 12 to 24 h.p.i., when viral mRNA is readily detectable (Panuska et al., 1990a, b), viral proteins were distributed diffusely throughout the cellular cytoplasm probably reflecting new protein synthesis. After 96 h, alveolar macrophages had differentiated and became markedly enlarged which yielded a more granular, diffuse cytoplasmic staining of viral proteins than that seen at earlier time points.

Importantly, the number of infected cells became maximal by 12 to 24 h.p.i. and remained nearly constant up to 96 h.p.i. consistent with prior results (Panuska et al., 1990a) and indicating that the RSV-permissive subpopulation of alveolar macrophages were stable. Several additional lines of evidence supported this observation. (i) Virus was produced by infected alveolar macrophages but did not spread to all cells in these cultures as is seen with RSV-permissive cell lines. (ii) The number of cells that replicated RSV decreased with increasing time of in vitro culture which made it unlikely that virus was continually transmitted from infected to uninfected cells. (iii) Repetitive infection or increased viral dose did not significantly alter either the number of

**Fig. 5.** Effects of TNFα dose on RSV replication. Alveolar macrophages (10⁶ cells/ml) were exposed to TNFα at the indicated doses for 16 h prior to infection with RSV at an m.o.i. of 3. At 24 h.p.i. cells were harvested and assessed for viral replication by ICA (see Methods). Results shown are mean ± s.d. for triplicate samples from a single donor and are representative of results seen for three separate donors.

Discussion

Previous studies examining transcriptional levels of viral RNA (Panuska et al., 1990b) and production of infectious virus (Panuska et al., 1990a; Becker et al., 1992) have demonstrated that human alveolar macrophages replicate RSV. However, these approaches do not readily permit characterization of the RSV-permissive subpopulation. Here we have examined RSV replication at the individual cell level employing immunofluorescence of viral proteins to detect infected cells and ICA to detect cells that replicate RSV. These approaches demonstrated that: a maximum number of alveolar macrophages were infected by 12 to 24 h.p.i. which remained constant up to 96 h.p.i.; HLA-DR-positive alveolar macrophages contained a discrete RSV-permissive subpopulation of cells; only a fraction of the infected cells replicated RSV; in vitro culture of alveolar macrophages resulted in a progressive decrease in the number of cells that replicated virus; and TNFα which is rapidly induced in RSV-infected alveolar macrophages (Panuska et al., 1990b; Becker et al., 1991) restricted viral replication without altering the number of infected cells when administered prior to infection.

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infected cells nor the percentage of cells capable of replicating virus. Together these results indicated that only a stable subpopulation of alveolar macrophages was permissive to RSV infection but the phenotypic characteristics of this subpopulation have not been previously defined.

One characteristic of the RSV-permissive subpopulation of alveolar macrophages included coexpression of HLA-DR molecules as defined by FACS analysis. Approximately 65% of our uninfected alveolar macrophages expressed HLA-DR molecules. This is in agreement with several previous studies (Rossi et al., 1986; Rich et al., 1987) but differs from other studies (Lipscomb et al., 1986; Nicod et al., 1989). The FACS criteria employed were designed to separate auto-fluorescence completely from PE fluorescence and may have underestimated the size of our HLA-DR-positive population accounting for these apparent differences. Nevertheless, our results indicated that RSV infection increased the density (amount) of HLA-DR molecules on RSV-infected alveolar macrophages whilst simultaneously resulting in a minor, but significant decrease in the number of HLA-DR-positive cells (Table 1). Blocking HLA-DR framework determinants did not reduce RSV infection of alveolar macrophages as has been suggested for other viruses (Inada & Mims, 1984; Mann et al., 1988).

Expression of HLA-DR molecules varies with the state of activation and differentiation of macrophages and is further regulated (both positively and negatively) by soluble cytokines such as interferon gamma and TNFα (Leiter et al., 1989; Watanabe & Jacob, 1991). Prior studies by our laboratory have indicated that when alveolar macrophages are infected in vivo with RSV this activates these cells to produce IL-1 and to coexpress HLA-DR and RSV proteins (Panuska et al., 1992). Expression of foreign (viral) proteins, HLA-DR molecules and IL-1 are the critical determinants regulating antigen-presenting functions (Unanue & Allen, 1987; Unanue & Cerrotini, 1989); therefore, alveolar macrophages may perform these functions in response to RSV.

We have previously shown that alveolar macrophages replicate RSV for 25 days p.i. (Panuska et al., 1990a). However viral production per infected cell progressively decreases from 0.05 p.f.u./cell at 24 h.p.i. to 0.003 p.f.u./cell by 240 h.p.i. Other authors have confirmed that RSV-infected alveolar macrophages produce low levels of infectious virus (Becker et al., 1992). The present studies offer a partial explanation for these findings. The number of infected, freshly isolated alveolar macrophages correlated well with the number capable of replicating RSV, indicating that viral protein expression and ICA accurately detected infected cells. Continued expression of viral proteins by a constant proportion of the alveolar macrophage population concomitant with a progressive decrease in the proportion of cells that replicated virus after in vitro culture suggests that most of these cells undergo abortive replication. The block in virus production may occur at virus maturation, assembly or release. Other explanations such as inactivation of extracellular virus, competition for virus-dependent substrates, or production of defective interfering particles were unlikely because supernatants from infected alveolar macrophages when mixed with stock RSV did not alter the expected titre. Furthermore, previous studies by our laboratory coupled with the present studies indicate that RSV does not result in cytolysis of infected alveolar macrophages (Panuska et al., 1990a). Thus, RSV replication may be restricted at multiple levels including endogenous cellular mechanisms as well as by autocrine or paracrine mechanisms.

The progressive decrease in viral replication with increasing time of in vitro culture of alveolar macrophages prior to infection contrasts with the findings from studies of monocytes where in vitro culture yielded more efficient RSV replication (Krilov et al., 1987). However, RSV induces TNF production by alveolar macrophages and not by blood monocytes (Panuska et al., 1990b) and therefore, differences in replication of RSV in these separate cells might be expected.

TNFα inhibits RSV infection of blood monocytes (Midulla et al., 1989) as well as other cell types (Wong & Goeddel, 1986; Mestan et al., 1986; Ahrens et al., 1990) and, as shown here, restricted RSV replication in alveolar macrophages although not as potently as in vitro differentiation. RSV rapidly induces TNFα expression by alveolar macrophages resulting in production of this cytokine up to levels comparable to those employed here (Panuska et al., 1990b; Becker et al., 1991). It was possible that alveolar macrophages were resistant to reinfection or higher viral doses due to progressive in vitro differentiation or possibly TNFα-mediated autocrine or paracrine restriction of RSV. Addition of excess neutralizing levels of anti-TNFα to our cultures did not affect viral replication (data not shown) suggesting that endogenous cellular mechanisms may account for the viral restriction. However, we interpreted these results cautiously because we could not exclude the possibility that TNFα could have interacted with its receptor prior to capture by the neutralizing antibody. It was also observed that TNF had no effects on RSV replication by CV1 cells suggesting that TNFα may affect the state of differentiation of macrophages as has been reported by other authors (Beutler & Cerami, 1989; Witsell & Schook, 1992).

A variety of cytokines and arachidonic acid metabolites are produced by alveolar macrophages in response to RSV. IL-1β, IL-6, IL-8, PGE₂, PAF and lyso-
PAF did not significantly alter RSV infection or replication in alveolar macrophages. In contrast, pretreatment with TNFα inhibited RSV replication in a time- and dose-dependent manner. Local production of TNFα in response to RSV could function to restrict RSV replication through autocrine/paracrine mechanisms. Since TNFα can promote in vitro macrophage differentiation (Beutler & Cerami, 1989; Witsell & Schook, 1992) it is possible that the mechanism by which TNFα restricts RSV replication may be through augmented macrophage differentiation.

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References


RSV replication in alveolar macrophages


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