Permissiveness of Guinea Pig Alveolar Macrophage Subpopulations to Acute Respiratory Syncytial Virus Infection

In Vitro*

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Background and objectives: Alveolar macrophages (AMs) are targets for respiratory syncytial virus (RSV) infection in vivo and in vitro. However, only a minority of AMs are permissive to acute RSV infection in vitro, and it is unknown whether this permissiveness may be related to the degree of cellular maturation that is achieved in vivo.

Methods: By using density gradient centrifugation, in which the degree of AM maturation is inversely related to buoyant density, we prepared three subpopulations of guinea pig AMs (designated as hypodense, intermediate-density, and high-density AMs). Twenty-four hours after exposure to RSV in vitro, the percentage of RSV-positive cells in each subpopulation was determined by immunocytochemistry; intracellular virus was released from cells by sonication and quantified by plaque assay, and intracellular localization of RSV proteins was evaluated by immunogold electron microscopy.

Results: High-density AMs had a significantly higher proportion of RSV-positive cells than hypodense AMs (p < 0.001), with intermediate-density AMs having intermediate values. The amounts of intracellular virus significantly increased from hypodense to intermediate density to high-density AMs (p < 0.001). Hypodense cells showed immunogold labeling principally within phagolysosomes, whereas intermediate-density and high-density cells showed immunolabeling of free cytoplasmic viral proteins and nucleocapsids.

Conclusions: The permissiveness of guinea pig AMs to acute RSV infection in vitro is inversely related to their degree of maturation achieved in vivo. In addition, these results suggest that immature, high-density AMs support RSV replication whereas more mature, hypodense AMs may restrict viral replication.

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Key words: alveolar macrophages; guinea pig; respiratory syncytial virus

Abbreviations: AM = alveolar macrophage; ATCC = American Type Culture Collection; FBS = fetal bovine serum; MEM = minimal essential medium; moi = multiplicity of infection; PBS = phosphate-buffered saline solution; RSV = respiratory syncytial virus; TBS = Tris-buffered saline

Respiratory syncytial virus (RSV) is a major cause of acute bronchiolitis and pneumonia in young children.1 The pathogenesis of acute bronchiolitis has generally been considered to involve RSV infection of airway epithelial cells,2 but more recently, several groups have established that RSV proteins also localize to alveolar macrophages (AMs) in vivo during acute bronchiolitis.3,4 Using a guinea pig model of experimental RSV infection, we have also demonstrated viral protein within AMs during acute bronchiolitis.5 AMs are cells derived from blood monocytes that protect the lower respiratory tract against pathogens (including viruses) and inhaled particles and maintain sterile conditions in the lung.6 Despite the role of AMs in host lung defense mechanisms, several studies have reported that RSV can cause productive infection of human AMs and blood monocytes in vitro.7–10 However, only a minority of these cells are permissive to RSV infection, even with exposures to large amounts of virus, and the reasons for this limited permissiveness of AMs to RSV infection are poorly understood. Moreover, the
compartmentalization of RSV proteins within infected AMs has not been evaluated by electron microscopy, such that our knowledge of virus-cell interactions at the ultrastructural level is limited.

It is well established that the population of AMs is normally heterogeneous and that subpopulations of these cells can be separated by density gradient centrifugation, in which the degree of cellular maturation achieved in vitro is inversely related to buoyant density.\textsuperscript{11,12} Since cord blood monocytes have been shown to be significantly more permissive to RSV infection than AMs,\textsuperscript{8} we hypothesized that the permissiveness of AMs to acute RSV infection in vitro may be inversely related to their degree of maturation achieved in vitro, where comparatively immature AMs would show increased permissiveness to the virus. To investigate this hypothesis, we performed BAL on guinea pigs and prepared subpopulations of AMs by centrifugation on discontinuous gradients of metrizamide. The permissiveness of these AM subpopulations to acute RSV infection in vitro was studied by using immunocytochemistry to enumerate cells containing viral proteins and viral plaque assays were used to quantify amounts of intracellular virus. To explore the intracellular localization of RSV proteins in the AM subpopulations after experimental RSV infection, we performed immunogold electron microscopy on ultrathin sections of glutaraldehyde-fixed cells.

**Materials and Methods**

**Virus**

The Long strain A of human RSV was obtained (American Type Culture Collection [ATCC]; Manassas, VA). RSV was propagated on HEp-2 cell monolayers (ATCC) in minimal essential medium (MEM) supplemented with 2% heat-inactivated fetal bovine serum (FBS) and 50 μg/mL gentamicin (Gibco; Grand Island, NY). When > 75% of cells exhibited cytopathic effect (formation of syncytia, cell rounding, and detachment), the culture flasks were shaken with autoclaved 3-mm glass beads and the resulting cell suspension was sonicated on ice for 1 min at the maximum output from a sonicator (Vibra Cell; Sonics & Materials Inc; Danbury, CT). Cellular debris was removed by centrifugation at 1,500 × g and 4°C for 15 min and the cleared supernatant containing virus was kept at −70°C and used for infection immediately after determining the titer by plaque assay.

**Animals**

Juvenile female Cam Hartley guinea pigs, 22 to 29 days old (250 to 300 g body weight), were purchased (Charles River Laboratories; Montreál, QC, Canada) and were housed as previously described,\textsuperscript{2} in conditions of plastic cages containing corn cob bedding; access to guinea pig chow (Purina;Ralston Purina Company; St. Louis, MO), alfalfa hay cubes and water, and 12-h alternating light-dark cycles. Animals were maintained in accordance with standards of the Canadian Council on Animal Care.\textsuperscript{3}

**BAL, Blood Collection, and Cell Processing**

Animals were anesthetized by intraperitoneal administration of pentobarbital (Euthanyl; MTC Pharmaceuticals; Cambridge, ON, Canada), exsanguinated by cardiac puncture, and 5 mL of blood was collected in EDTA-containing tubes. Blood mononuclear cells were isolated from whole blood by standard methods using Ficoll-paque density gradient centrifugation (Pharmacia Biotech; Uppsala, Sweden). BAL was performed in situ by instilling intratracheally, 5 to 10 mL aliquots of sterile, nonglycogen normal saline solution, prewarmed at 37°C (total volume: 100 mL). After each instillation, the fractions were gently aspirated and the fluids were pooled in 50-mL centrifuge tubes and kept on ice until processed. Typically, the percent recovery of lavage fluid by this method was > 95% and no blood contamination occurred during the lavage procedure. BAL cells were sedimented by centrifugation at 500 × g for 10 min at 4°C, and counted in an automated cell counter (Coulter Electronics Inc; Hialeah, FL). More than 85% of total cells recovered by lavage were AMs (mean ± SD: 87.8 ± 2.6%). Cell viability, as determined by trypan blue dye exclusion, was >95%.

**Metrizamide Gradient Density Centrifugation**

Discontinuous gradients of metrizamide (Nycomed Pharma AS; Oslo, Norway) were prepared from 27% metrizamide stock solution diluted with 10 mM HEPES-buffered saline solution at a pH of 7.3 containing 0.1% each of glucose and gelatin. The method was derived from Fukuda et al,\textsuperscript{14} and Vadás et al,\textsuperscript{15} in which metrizamide-containing solutions are isotonic. Up to 107 BAL cells were resuspended in 2 mL of HEPES-saline solution and layered on top of increasing concentrations of metrizamide gradients (3 mL at 27%; and 2 mL each at 22%, 20%, and 18% metrizamide) in 15-mL transparent polypolyethylene centrifugation tubes (Corning; Corning, NY). After centrifugation at 1,200 × g for 45 min at 18°C, the cells were collected from each interface, washed with Hanks’ balanced salt solution containing 2% FBS and were counted before being resuspended in MEM supplemented with 5% FBS. The total cell recovery after density gradient centrifugation ranged from 70 to 75%.

**Preparation of AM Subpopulations**

Results of preliminary experiments, in which BAL cells were fractionated on several layers of metrizamide (16%, 18%, 20%, 22%, and 27%), indicated that > 95% of total AMs recovered after centrifugation localized to the interfaces of gradients < 22% metrizamide. The 22% metrizamide cutoff value was based on the distribution of blood monocytes that were used as a reference for buoyant density (see below). The cells recovered from the top of the 16% metrizamide layer had a similar morphology to those recovered from the 16 to 18 layer, and these were therefore combined in subsequent experiments. Overall, we prepared three subpopulations of AMs from interfaces as follows: < 18%; 18 to 20%; 20 to 22% metrizamide. The macrophage phenotype of cells in the three isolated populations was confirmed by the alkaline phosphatase antialkaline phosphatase method, by using MR-1 monoclonal antibody (Serotec Ltd; Mississauga, ON, Canada) that recognizes a cytoplasmic component of guinea pig macrophages and monocytes\textsuperscript{6} (Fig 1). Using the same centrifugation procedure as for BAL cells, blood monocytes predominantly localized to the 20 to 22% metrizamide interface and were not present in the lower-density gradients. Accordingly, we designated the subpopulation of AMs recovered at the interface 20 to 22% metrizamide as “high-density” AMs; cells recovered at the interface 18 to 20% metrizamide were called “intermediate-density” AMs; and those collected on top of the 18% metrizamide
were manually agitated were exposed well in hundred intermediate-density, recovered AMs for 37°C as confirmed ing adherent removing nonadherent cells had vacuolated latter fraction, for gradient approximately 10^6 Vitro In high mic ratio; (24%).

The isolated cells were counted and their number adjusted to approximately 10^6 macrophages (monocytes) per milliliter. Five hundred microliters of each cell suspension were added to each well in six-well culture plates and the cells were allowed to adhere for 60 min at 37°C in 5% CO2-containing atmosphere. After removing nonadherent cells by washing with MEM, the remaining adherent cells (ie: all of which were macrophages/monocytes, as confirmed by MR-1 antibody staining of designated aliquots) were exposed to RSV at a multiplicity of infection (moi) of 1 at 37°C for 90 min. During this incubation period, the culture plates were manually agitated every 15 min to ensure exposure of cells to the virus. After two washes with MEM to remove nonadsorbed virus, the infected cultures were maintained at 37°C for 24 h before processing for RSV immunocytochemistry, plaque assays, and immunogold transmission electron microscopy. The moi and duration of culture postinfection were chosen based on our preliminary RSV immunostaining of virus-exposed, unfractionated BAL cells, in which similar percentages of RSV-positive AMs were observed at moi = 1 or 2 (mean ± SD: 26.5 ± 3.1% vs 28.5 ± 4.6%, respectively) at 24 h postinfection, with no further increase by 48 h. Cells were examined for evidence of detachment from plastic 24 h after in vitro exposure to RSV, and AM viability was assessed by trypan blue dye exclusion.

**RSV Immunostaining**

After aspirating the culture media, the cells were fixed in the wells for 10 min with 4% paraformaldehyde in phosphate-buffered saline solution (PBS), rinsed with PBS, and harvested by gently scraping with a sterile rubber policeman. Cytospin slides were then prepared from the collected cell suspensions. RSV proteins were detected by indirect immunoperoxidase staining using a primary rabbit polyclonal anti-RSV antibody and a
Viral Plaque Assays

At the end of the incubation period, the infected cells were harvested from each well by gentle scraping with an autoclaved rubber policeman, counted and placed in sterile eppendorf tubes before centrifugation at 500g for 5 min at 4°C in a microcentrifuge. The cells in the pellet were resuspended in 1 mL of cold MEM and sonicated on ice for 30 sec to release cell-associated virus. The amounts of released virus were quantified by plaque assay, with RSV-specific cytopathic effect (syncytia) further confirmed by immunostaining using anti-RSV antibody, as previously described.27 Results of plaque assays were expressed as numbers of plaque forming units (pfu) per 106 cells.

Immunogold Electron Microscopy

After exposure to RSV, as described above, AMs were fixed in 2.5% glutaraldehyde-containing sodium cacodylate buffer (0.1 M, pH 7.4) for 1 h at 4°C, postfixed in osmium tetroxide for 1 h at room temperature, and embedded in epoxy resin following standard methods.18 Ultrathin sections were cut with an ultramicrotome (Reichert; Vienna, Austria) and collected onto Formvar vinyl alcohol-vinyl acetate formal copolymer-coated nickel grids (Ted Pella, Inc; Redding, CA). Following treatment with sodium metaperiodate to unmask antigenic sites28 and blocking nonspecific binding by incubation with normal rabbit serum (5% in PBS, 20 min), the sections were incubated with a cocktail of mouse anti-RSV monoclonal antibodies optimally diluted 1:100 as recommended by the manufacturer (NCL-RSV2; Novacastra Laboratories; Newcastle Upon Tyne, UK). The sections were washed again with PBS and then incubated at room temperature for 30 min with a rabbit antirabbit antibody (1:200) conjugated to 15 nm colloidal gold particles (Dako). After three washes in PBS, followed by a final wash in distilled water, the sections were contrasted with uranyl acetate and lead citrate.29 For control sections, incubation with the primary antibody was omitted. Sections were examined under a transmission electron microscope (Philips 400; N. V. Philips’ Gloeilampen-fabrieken; Eindhoven, The Netherlands).

Statistical Analysis

The data are presented as means ± SD of values obtained from five guinea pigs. Data were analyzed using a one-way analysis of variance, and a sequential rejective Bonferroni procedure was used to correct for multiple comparisons. A p value < 0.05 was considered to be statistically significant.

RESULTS

Viability of AM Subpopulations After In Vitro Exposure to RSV

Hypodense and intermediate-density AMs showed no apparent loss in cell adhesiveness to the culture plates at 24 h postinfection with RSV. Some detachment (< 10% of adherent cells) occurred at this time point in RSV-exposed high-density AMs and in RSV-exposed blood monocytes. However, cell viability remained > 95% in all cultures at 24 h postinfection.

RSV Immunostaining

Figure 2 shows typical cytoplasmic localization of RSV proteins within isolated AMs in the three subpopulations, as detected by immunocytochemistry at 24 h postinfection. Positive RSV immunostaining within hypodense AMs showed a distinct granular pattern within the cytoplasm, whereas a less granular and more diffuse cytoplasmic immunostaining pattern was observed for intermediate-density and high-density cells. High-density AMs showed two-fold higher proportion of RSV-positive cells than hypodense AMs. As summarized in Figure 3, hypodense AMs showed the lowest percentage of RSV-positive cells in comparison to the other subpopulations of AMs and blood monocytes similarly exposed to RSV in vitro (p < 0.01). The percentage of RSV-positive cells within the high-density AM subpopulation was approximately two-fold higher than the hypodense AM subpopulation (20.6 ± 2.9% vs 10.6 ± 1.5%, respectively, p < 0.001). The percentage of RSV-positive intermediate-density AMs (18.6 ± 2.1%) was similar to that of high-density AMs, but was significantly lower than that of blood monocytes (25.2 ± 4.5%, p < 0.01). In specimens of unfractionated BAL cells, the predominantly granular pattern of cytoplasmic immunostaining was observed within large vacuolated cells, while smaller cells showed a more diffuse staining pattern, similar to results observed with hypodense and high-density fractions, respectively.

Viral Plaque Assays

Figure 4 illustrates the amounts of intracellular virus isolated by sonication from the different AM subpopulations at 24 h postinfection. The amounts of isolated virus increased with the buoyant density of AMs (hypodense AMs < intermediate density...
AMs < high-density AMs, p < 0.001), with high-density cells showing 18-fold greater amounts of RSV in comparison to hypodense AMs (4,554 ± 1,680 vs 252 ± 61 plaque-forming units/10⁶ cells, respectively). No statistically significant difference was observed between high-density AMs and blood monocytes in the amounts of virus isolated at 24 h post-RSV infection (p = 0.15).

**Immunogold Electron Microscopy**

Figure 5 shows representative electron micrographs of RSV-exposed AMs immunolabeled with the pool of monoclonal anti-RSV antibodies. There were apparent differences between the three AM subpopulations in the patterns of cytoplasmic distribution of colloidal gold particles. Hypodense cells showed aggregates of colloidal gold particles within phagolysosomes (Figure 5, A), consistent with the granular pattern of RSV immunostaining observed by light microscopy. By contrast, intermediate- and high-density AMs (Fig 5, B and C) showed colloidal gold particles free within the cytoplasm, frequently in a perinuclear distribution that has been described for RSV,²² and in association with typical RSV nucleocapsids.²³ Examination of control sections (Fig 5, D) revealed rare single colloidal gold particles that did not show a particular distribution within cells.

**DISCUSSION**

The overall objective of this study was to determine whether the permissiveness of normal guinea pigs’ AMs to acute RSV infection in vitro may be related to their degree of cellular maturation achieved in vivo. We prepared three populations of...
AMs based on previous reports that showed an inverse relationship between cellular maturity and buoyant density, in which high-density AMs had similar properties to blood monocytes from which AMs differentiate.\textsuperscript{11,12} Our results confirmed these previous findings and extended them by showing that comparatively immature, high-density AMs had a significantly higher percentage of RSV-positive cells than more mature, hypodense AMs, with intermediate values obtained for intermediate-density AMs. In addition, the results of viral plaque assays revealed that the amounts of intracellular virus were significantly increased from hypodense to intermediate-density to high-density AMs, with high-density AMs and blood monocytes showing similar amounts. Importantly, high-density AMs had a two-fold greater percentage of RSV-positive cells than hypodense AMs by immunocytochemistry but showed 18-fold more replicating virus by plaque assays. Taken together, these findings demonstrate that the permissiveness of AMs to acute RSV infection is inversely related to their degree of maturity and suggest that high-density AMs support RSV replication whereas hypodense AMs may restrict RSV replication after internalization of the virus.

Further analysis by immunogold labeling provided qualitative information about patterns of virus-cell interactions that may be pertinent to the ability of the different AM subpopulations to support or restrict viral replication. In particular, immunolabeling within the phagolysosomes of hypodense AMs correlates with the granular staining pattern observed under light microscopy and is consistent with this subpopulation of cells preferentially inactivating the virus. In contrast, the distribution of gold particles free within the cytoplasm and in association with viral nucleocapsids may be related to the greater tendency of intermediate-density and high-density cells to support viral replication.

Restriction of \textit{in vitro} RSV replication has been reported previously in unfractionated human AMs obtained from adults.\textsuperscript{8} The mechanisms of this restricted viral replication in adults remain unclear although they could involve production of cytokines such as tumor necrosis factor-\(\alpha\) by infected cells.\textsuperscript{13,24} The results of this study suggest that restriction of RSV replication in humans is likely modulated by the subpopulation of hypodense AMs, where compartmentalization of virus within phagolysosomes may be involved in addition to virus-induced production of cytokines.

We speculate that these differences in RSV-cell interactions between AM subpopulations, reported in this study, may be an important factor that could determine the susceptibility of an individual to develop severe or limited RSV lower respiratory tract infections. Since most AMs in the lungs of normal adults are hypodense,\textsuperscript{25} effective restriction of viral replication by these cells might confer protection that limits the severity of RSV infection. By contrast, the relative proportions of high-density and hypodense AMs in the alveolar space of children are
unknown, and if children have a predominant population of high-density AMs, then this milieu might support increased viral replication and further contribute to more severe lower respiratory tract infections.

In summary, this study has demonstrated that the permissiveness of AM subpopulations to acute RSV infection in vitro is inversely related to their degree of maturation achieved in vivo, where comparatively immature, high-density AMs support viral replication and mature, hypodense AMs may restrict viral replication. An improved understanding of the mechanisms responsible for these different virus-cell interactions may help to develop innovative approaches for the prevention and treatment of severe RSV lower respiratory tract infections.

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REFERENCES


