Previous attempts to culture mouse alveolar type II (ATII) cells have been hampered by limited purity and cell recovery. We have now obtained culturable ATII cells from female C57BL/6 mice at a purity of 92% ± 3 (mean ± SD; n = 20), with viabilities of 96% ± 2 and total yields of 5.1 ± 0.7 x 10^6 cells per mouse. Crude lung cell suspensions were prepared by intratracheal instillation of Dispase and agarose followed by mechanical disaggregation of the lungs. Crude cell suspensions were purified by negative selection using a biotinylated-antibody, streptavidin-coated biomagnetic particle system. Cell purities were determined by Pap staining and confirmed ultrastructurally. Purified ATII cells were cultured on fibronectin-coated chamber slides and maintained for up to 5 days in DMEM with 10% fetal bovine serum. Cultures exhibited minimal contamination by Clara cells, mesenchymal cells, or endothelial cells, and the epithelial nature of the cultures was confirmed by positive cytokeratin staining in at least 97% of the cells through day 5. Day 3 cultures demonstrated osmium tetroxide/tannic acid-stained granules consistent with lamellar bodies in 76% ± 3.6 of the cells. The cultures displayed features distinct from those previously described for adult rat ATII cells, including irregularly-shaped cells and the formation of numerous cytoplasmic projections in direct contact with other cells. These studies indicate that excellent yields of highly purified, culturable ATII cells can be obtained from genetically defined mice. These techniques may provide powerful new models for the study of parenchymal lung disease in vitro.

In vivo mouse models have been used to study the pathobiology of the lung for many years. Investigations of the effects of particulates (1, 2), antibiotics (3, 4), hyperoxia (5), ionizing radiation (6, 7) and chemical-induced injury (8, 9) on murine pulmonary pathobiology have made significant contributions to our understanding of many disease processes in the lung. In vivo mouse models continue to be important in testing the genetic bases for lung disease. For example, comparisons of genetically defined strains of mice demonstrate that several exhibit differential susceptibilities to pulmonary disease (7, 9-11). Recent advances in transgenic technology in mice have also provided powerful tools for the investigation of lung diseases (12).

Technical difficulties in purifying murine ATII cells have limited the development of corresponding in vitro models to complement the existing in vivo models of lung disease. In vitro models from other species have contributed a great deal to the understanding of ATII cell biology. Similar in vitro systems that exploit existing murine in vivo models of lung disease, genetically defined strains of mice, and transgenic technology will open new venues for investigating lung disease.

Previous methods for isolating murine ATII cells have suffered from poor purity, cell recovery, and viability. Massey and Geddes used centrifugal elutriation to isolate mouse ATII cells to 65% purity with final yields that were typically less than 1 million cells per mouse (13). Kumar and colleagues reported better yields (approximately 2 million cells per mouse) using velocity sedimentation and differential attachment affinity to IgG-coated plates to separate ATII cells, but purities were less than 65% (14). Our laboratory previously developed a method for purifying the cells to 95% by laser flow cytometry (15); however, final yields were less than 2 million cells per mouse and the cells obtained were not suitable for culture.

This paper describes a new method for obtaining highly purified, viable ATII cells from mice in high yields, which can then be cultured. When cultured on fibronectin, murine ATII cells demonstrated features typical of ATII cells, including intracellular lamellar bodies by osmium tetroxide/
tannic acid staining. We expect that these techniques will allow new data to be obtained from established in vivo murine models for distal lung disease.

Materials and Methods
Reagents and Antibodies
Mice were obtained from Charles River Laboratories, Wilmington, MA; pentobarbital (Nembutal) from Abbott Laboratories (North Chicago, IL); intravenous catheters (Jelco) from Critikon, Inc. (Tampa, FL); Dispase from Collaborative Research, Inc. (Bedford, MA); low melt agarose, Dulbecco’s modified Eagle’s media (DMEM) with high glucose with glutamine, HEPES buffer, deoxyribonuclease I (DNase I) type II, heat inactivated fetal bovine serum, mouse-IgG, fibronectin, penicillin/streptomycin, and anti-vimentin (vim.13.2) from Sigma (St. Louis, MO); Falcon cell strainers from Becton Dickinson (Franklin, NJ); nylon mesh from Teko, Inc. (Lancaster, NY); biotinylated rat anti-mouse anti-CD-32 and biotinylated rat anti-mouse anti-CD-45 from Pharmingen (San Diego, CA); streptavidin-coated Magnespheres from Promega (Madison, NJ); magnetic tube separator from Perceptive Diagnostics (Cambridge, MA); eight-well permanox chamber slides from Nunc, Inc. (Naperville, IL); anti-cytokeratin 8/18 (cam5.2) from Becton Dickinson Immunocytochemistry Systems (San Jose, CA); anti-desmin (D33) and the Dakopatts LSAB-II Immunohistochemistry Staining Kit from Dako (Carpinteria, CA); triple concentrated hematoxylin, aqueous mounting media (Aquamount), and Gill Biological Nuclear Stain from Lerner Laboratories (Pittsburgh, PA).

Media
DMEM was prepared with 25 mM HEPES buffer. Culture medium was prepared with 10% fetal bovine sera, and 1% penicillin-streptomycin in 25 mM HEPES buffered DMEM.

Preparation of Crude Single Lung Cell Suspensions
Crude cell suspensions were prepared from female, 18- to 22-g C57BL/6 mice using a modification of our previous method (15). The mice were sedated with pentobarbital intraperitoneally (50 mg/ml, 3.25 µl/g body weight), secured to a dissecting board, and exsanguinated by opening the peritoneum and clipping the left renal artery, and the ventral rib cage was removed. The lungs were perfused with 0.9% NaCl, using a 10-ml syringe fitted with a 21-gauge needle, through the right ventricle of the heart until they were visually free of blood. A 20-gauge intravenous catheter was inserted into the trachea and secured tightly with a suture. The lungs were filled with 1 to 2 ml Dispase via the tracheal catheter and then allowed to collapse naturally, expelling part of the Dispase. Low melt agarose (1%, 0.45 ml, stored in a 45°C water bath) was infused slowly via the catheter. The lungs were immediately covered with crushed ice and incubated for 2 min. The lungs were then removed to 2 ml Dispase in a 12-ml polypropylene culture tube, incubated for 45 min at room temperature, and placed on ice until the next step. The lungs were transferred to 7 ml DMEM with 0.01% DNase I in a 60-mm Petri dish. The digested tissue was carefully teased from the airways with the curved edge of curved fine-tipped forceps and gently swirled for 5 to 10 min. The resulting suspension was successively filtered through 100-µm and 40-µm Falcon cell strainers, and then through a 25-µm nylon mesh. The filtered suspension was centrifuged at 130 × g for 8 min at 4°C and resuspended in 10 ml of culture media.

Magnetic Purification of ATII Cells from Crude Cell Suspensions
The cells were incubated with biotinylated anti-CD-32 (0.65 µg/million cells) and biotinylated anti-CD-45 (1.5 µg/million cells) for 30 min at 37°C. Meanwhile, streptavidin-coated magnetic particles were washed twice in phosphate buffered saline (PBS) (10 min each wash) in a polypropylene culture tube using a magnetic tube separator. After incubation, the cells were centrifuged (130 × g for 8 min at 4°C), resuspended in 7 ml DMEM, added to the magnetic particles, and incubated with gentle rocking for 30 min at room temperature. At the end of the incubation, the tube was attached to the magnetic tube separator with adhesive tape for 15 min. The cell suspension was aspirated from the bottom of the tube using a narrow-stemmed transfer pipet, centrifuged, and resuspended in culture media. Viabilities were determined by trypan blue exclusion.

Preparation of Clara Cell Suspensions from Mice
Crude cell suspensions were prepared using a modification of the ATII cell purification technique described earlier, as follows: after the tracheal catheter was secured, the lungs were instilled with 1 ml low-melt agarose. Dispase (0.8 ml at 37°C) was immediately instilled while ice was simultaneously placed over the lungs. After 2 min, the ice was removed and the lungs were incubated in place for 45 min. The lungs were removed to a Petri dish with DMEM and DNase (as described earlier), teased apart, swirled for 10 min at room temperature, and filtered through 100- and 40-µm mesh. The suspension was centrifuged (130 × g, 8 min, 4°C) and resuspended in DMEM. Crude cell suspensions were purified by previously described methods using IgG panning (17). The purity of the suspensions was 75 to 80% as determined by nitroblue tetrazolium (NBT) staining (as described subsequently) of cytocentrifuged preparations.

ATII and Clara Cell Cultures
For each culture, the day of the purification was designated culture day 0. Purified ATII and Clara cells were suspended in 3 ml culture medium and incubated for at least 4 h to a maximum of 16 h in tissue-culture-treated 60-mm Petri dishes to remove residual mesenchymal cells. Suspensions were centrifuged (8 min at 130 × g, 4°C) and resuspended to 1 to 1.5 million cells per milliliter. Three hundred microliters of each suspension was added to plastic chamber slide wells (8 wells per slide, 1 cm² each well) that were precoated as per the manufacturers instructions with fibronectin (10 µg/cm²). Cultures were incubated in a humidified, 10% CO₂ chamber at 37°C and observed daily by phase contrast microscopy. On days 2, 3, or 5, cultures were washed twice (see the subsequent discussion), fixed appropriately (see as described subsequently) and stained with NBT and osmium tetroxide/tannic acid, or stained immunocytochemically for vimentin, desmin, or cytokeratin.
Mouse Mixed Lung Cell Cultures
Cultures were prepared by previously described methods (18) with one modification: airways were not cut away from lungs before mincing. After the cells reached confluence, cultures were trypsinized, filtered through 100-μm cell strainers, centrifuged, resuspended, replated onto fibronectin-coated 8-well plastic chamber slides and incubated until the cultures were confluent.

Pap Staining
Aliquots (1.5 × 10^5 cells) from crude and purified cell suspensions were diluted in 200 μl cacodylate buffer (1M), centrifuged onto glass slides with a Shandon Cytospin Centrifuge at 600 rpm for 3 min, air dried, and stained within 3 h using a modified Pap stain (16).

Electron Microscopy
Pellets of purified ATII cells (approximately 5 million cells) were resuspended in a minimal volume of supernatant before fixing with 1 to 2 ml glutaraldehyde (3% in 0.1 M phosphate buffer) overnight at 4°C. The fixed suspensions were centrifuged and resuspended in 50 μl agarose (4%, low-melt). The agarose-embedded cells were postfixed with 1% osmium tetroxide, dehydrated through ethanol, and embedded in Epon using standard techniques. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy.

Immunocytochemistry
Cultures were washed with PBS, fixed with 95% ethanol, and stored at 4°C until stained. Cultures were stained for cytokeratin, vimentin, or desmin with a linked streptavidin-biotin immunoperoxidase system using the Dako LSAB II kit, following the manufacturer's directions with slight modifications. Samples were incubated for 1 h with primary antibodies, 45 min with secondary antibody, and 35 min with streptavidin conjugate. Slides were counterstained with tripple concentrated hematoxylin for 10 to 15 s and mounted using aqueous mounting media.

Osmium Tetroxide/Tannic Acid Staining
Slides were cooled to 4°C, washed twice with 1 M cacodylate buffer (4°C), fixed, and stained by a modification of previously described methods (19). All solutions were prepared with cacodylate buffer and maintained at 4°C unless otherwise specified. Slides were fixed in 1.5% glutaraldehyde (20 min), rinsed twice with cacodylate, and postfixed with 1% osmium tetroxide overnight. The slides were rinsed twice with cacodylate and once by immersing in cacodylate for 5 min. Just enough tannic acid (1%, pH 6.8) was applied to cover the fixed cultures. Slides were loosely coverslipped and incubated at room temperature until they were almost dry and staining of lamellar bodies was clearly visible (16 to 24 h). Coverslips were removed and slides were rinsed with water and mounted with aqueous mounting media.

Nitroblue Tetrachromium Staining
Day 2 ATII and Clara cell cultures were washed twice with PBS, air dried, fixed, NBT stained by previously described methods (17), and mounted with aqueous mounting media.

Cell Identification and Counting
The total numbers of cells in crude and purified cell suspensions in each of 20 samples were determined by hemocytometer counting. For Pap-stained slides, 500 cells from each slide were scored for the presence of numerous dark cytoplasmic granules.

Electron micrographs and corresponding Pap-stained slides were prepared individually for purified samples from four mice and evaluated for ATII cells. Only intact cells with nuclei were scored on electronmicrographs. All cells containing cytoplasmic organelles consisting of concentrically organized or layered membranes were considered ATII cells.

Type II cells were identified in osmium tetroxide/tannic acid-stained cultures by the presence of dark, lamellated cytoplasmic inclusions. Clara cells were identified in NBT-stained cultures by the presence of diffuse reticular cytoplasmic staining. Cytokeratin-, vimentin-, and desmin-positive cells were identified by the presence of positive-staining cytoplasmic filaments. Clara cell cultures served as positive controls for NBT staining. Mixed lung cell cultures served as positive controls for vimentin and desmin immunostaining. Each stain was repeated on ATII cell cultures individually prepared from three mice. Five hundred cells per culture were scored for specific positive or negative staining, and averages with standard deviations were calculated.

Results
Enzymatic digestion with Dispase and disaggregation of the lungs yielded crude cell suspensions containing 23.4 ± 4.2 million cells per mouse (n = 20). Pap staining of cytocentrifuge preparations of these cells revealed that 40.6% ± 5.9 were ATII cells. Purification via magnetic-bead-based negative selection yielded final suspensions of 5.1 ± 0.7 million cells with 92.0% ± 3.1 ATII cells by Pap staining (Figure 1A). In the final suspensions, 96.1% ± 1.8 of the cells were viable by vital dye exclusion. To confirm the purity assessment based on Pap staining, aliquots of purified ATII cells from four additional mice were prepared for both Pap staining and ultrastructural analysis. ATII cell purity by Pap staining in these experiments was 92.8% ± 2.8 (500 cells counted per mouse). Ultrastructural analysis confirmed these results, indicating that 95.0% ± 6.2 of the cells were ATII cells, based on content of characteristic lamellar bodies (43.5 ± 5.3 cells counted per mouse; Figure 1B).

Preliminary trials demonstrated that ATII cells from mice did not adhere to tissue-culture-treated plastic. However, contaminating mouse mesenchymal and endothelial cells attached firmly to plastic within 16 h (data not shown). Thus an initial incubation over plastic was done to improve purity of ATII cell cultures. Subsequently, the unattached ATII cells were transferred to fibronectin-coated surfaces, to which they attached within 24 h.

The cultures were evaluated for the presence of Clara cells by NBT staining, or for mesenchymal cells (vimentin or desmin), endothelial cells (vimentin), or epithelial cells (cytokeratin) by immunostaining. Since NBT staining of cultured Clara cells diminishes rapidly after attachment (17), ATII cultures and Clara cell control cultures were stained with NBT shortly after Clara cells in control cultures had completely attached and spread (48 h after isolation). Only
3.7% of the cells stained positively for NBT, compared with 72% in Clara cell control cultures, indicating minimal Clara cell contamination in ATII cell cultures. On day 3, desmin staining in ATII cultures was uniformly negative, and only 1.3% of the cells stained positively for vimentin, compared with 54% and 100% positive staining in respective controls (Table 1). On the other hand, 98.3% of the cells stained positively for cytokeratin, demonstrating that the cultures were predominantly composed of epithelial cells (Table 1). On day 5, 97% of the cells continued to stain positively for cytokeratin, and only minimal increases in mesenchymal or endothelial cells were observed (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Cytological Stain</th>
<th>Stain Specificity</th>
<th>Positive Control Cultures</th>
<th>Day (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osmium/tannic acid</td>
<td>ATII cells</td>
<td>-</td>
<td>76.0 ± 3.6%</td>
</tr>
<tr>
<td>Cytokeratin immunostaining</td>
<td>Epithelial cells</td>
<td>-</td>
<td>98.3 ± 0.9%</td>
</tr>
<tr>
<td>Nitroblue tetrazolium</td>
<td>Clara cells (72% positive)</td>
<td>3.7 ± 1.5%</td>
<td>-</td>
</tr>
<tr>
<td>Vimentin immunostaining</td>
<td>Fibroblasts and endothelial cells</td>
<td>-</td>
<td>1.3 ± 0.1%</td>
</tr>
<tr>
<td>Desmin immunostaining</td>
<td>Myofibroblasts and smooth muscle</td>
<td>-</td>
<td>0.0 ± 0.0%</td>
</tr>
</tbody>
</table>

*Data are means of % positive in 500 cells ± SD.
The morphology of the mouse ATII cell cultures was observed over 5 days by phase-contrast microscopy and by light-microscopic examination of fixed, osmium tetroxide/tannic acid-stained cultures on days 3 and 5. The cells attached lightly within 24 h after plating onto the fibronectin substrate. By 3 days after the initial isolation, a subpopulation of cells flattened and spread to form a monolayer (approximately 75% confluent), whereas other cells detached from the substrate. Cytoplasmic granules consistent with lamellar bodies were observed in 76% of the attached cells.

Figure 2. Day 3 cultures of purified mouse ATII cells visualized by osmium tetroxide/tannic acid-staining. Bars = 20 μm. A. Lamellate organization within osmium tetroxide/tannic acid-stained cytoplasmic granules is consistent with lamellar body structure. B. Lamellar bodies are present in 76% ± 3.6 of the cells in day 3 cultures (n = 3). C. Numerous cytoplasmic projections are visible above the plane of the monolayer. The projections appear to be in direct contact with other cells. D. Cytoplasmic processes are often branched. Intermediate and terminal bulges containing lamellar bodies are typically observed within attenuated cytoplasmic projections. E. Lamellar bodies are occasionally seen within attenuated areas of cytoplasmic projections. F. Irregularly shaped cells with nucleated plates connected to non-nucleated plates via thin extensions are reminiscent of ATII cells in vivo.
by osmium tetroxide/tannic acid staining (Table 1; Figure 2A and B). Many of the cells had developed cytoplasmic projections, apparently in direct contact with other cells (Figure 2C), which continued to be observed through day 5. Cytoplasmic projections were not restricted to the substrate but were observed above the plane of the monolayer (Figure 2C). Projections were often branched, with intermediate or terminal bulges (Figure 2C and D). Lamellae bodies were observed within the bulges (Figure 2C and D) or within the projections (Figure 2E). On day 3, many of the cells were irregularly shaped, occasionally with spread, nonnucleated areas connected to nucleated areas by thin cytoplasmic connections (Figure 2F). By day 5 the cells had spread to a flattened polygonal appearance, forming a confluent monolayer, with less than 14% of the cells retaining lamellae bodies by osmium tetroxide/tannic acid staining (Table 1). However, the loss of lamellae body content was not due to an overgrowth in nonepithelial cells, as evidenced by positive cytokeratin staining in 97% of the cells and minimal desmin or vimentin staining (Table 1).

Discussion

Mouse alveolar type II cells have been purified previously using elutriation (13) or a combination of velocity sedimentation and IgG panning (14). Although these methods yielded viable cells, the final preparations were only 65% ATII cells, yielding mixed cultures that have not been useful for functional studies. Mouse type II cells have been purified to 95% in our lab by flow cytometric sorting (15). However, the technique is expensive and time consuming, final yields are less than 2 million cells per mouse, and the cells have poor viability. The method described in this paper yields relatively large numbers of highly purified, viable mouse ATII cells suitable for culturing.

The main contaminating cell type in preparations made by previous methods for ATII purification was Clara cells (14). Because Clara cells have similar sizes, buoyant densities, and attachment behavior to ATII cells, and because of their abundance in mice, they have previously been difficult to separate from ATII cells. Our use of agarose to prevent Clara cell release into crude lung cell suspensions has been described previously (15), and we have refined it in the method described in this report to essentially eliminate Clara cells from crude preparations.

Mouse ATII cell isolation methods have previously employed nonspecific proteases such as type I protease (13) or trypsin (14). Nonspecific proteases can digest pulmonary interstitial proteins, releasing mesenchymal cells and reducing ATII cell purity, or can digest important cell surface proteins that may alter cell function. Although elastase has been widely and successfully used to isolate ATII cells from other species (16), variability between elastase preparations has been problematic. Dispase, in contrast, specifically cleaves type IV collagen and fibronectin, two of the major components of the basement membrane of type II epithelial cells, while only minimally interacting with type I collagen (20). Because of its enzymatic specificity, it is potentially more specific for releasing epithelial cells than the other proteases, and would not be expected to significantly interact with surface proteins that could alter cell characteristics or viability. Because the activity of Dispase is reduced by dilution, the lungs of the mice were not lavaged to remove macrophages prior to digestion. Instead, most contaminating cell types in the crude suspensions were labeled with biotin, using antibodies specific for cell surface proteins, and were subsequently removed using streptavidin-coated magnetic beads. Macrophages were labeled with an anti-mouse-Fc receptor (CD32) antibody, and other leukocytes were labeled via an anti-leukocyte (Common Leukocyte Antigen, CD45) antibody. Specific negative selection of contaminating cells has the advantage that ATII cells are not directly subjected to agents that may cause undesirable functional effects.

ATII cell yields from rats and rabbits typically range from 20 to 35 million cells per >200-g animal (16). Using the method described herein, yields of 4 to 7 million cells can be obtained from 20-g mice. Relative to the size of the animal, these yields compare favorably to those from the larger species. The yields obtained by this method are also 2- to 3-fold higher than those previously reported for isolated murine ATII cells (13-15).

Cultured rat ATII cells adhere to plastic, spread to a flattened polygonal appearance, and form monolayers with a uniform “cobblestone” pattern within 48 h (21). Unlike rat ATII cells, mouse ATII cells did not adhere to plastic. Therefore, after an initial incubation over plastic to remove other cell types, mouse ATII cell cultures were maintained on fibronectin-coated surfaces. Fibronectin has been shown to mediate rat ATII cell adherence to the substratum in vivo (22). Fibronectin also promotes greater attachment with faster spreading and more rapid loss of lamellae bodies compared with rat ATII cells grown on plastic surfaces (23, 24). In contrast, mouse ATII cells cultured on fibronectin attached and spread slowly, and retained lamellae bodies in 75% of cells for at least 48 h after plating (Figure 2A and B). The cells were often irregularly shaped (Figure 2D through F), rather than polygonal, and frequently displayed cytoplasmic projections extending to neighboring cells (Figure 2C through E).

The distinct morphologic appearance of cultured murine ATII cells may have functional significance. For example, the formation of cytoplasmic projections may play a role in ATII cell differentiation, injury repair, or cell-cell communication in vivo. Cytoplasmic projections on ATII cells have been observed previously in vivo and in vitro. Basolateral cytoplasmic projections from ATII cells extend through breaks in basement membranes in vivo, establishing direct contact with mesenchymal cells (25). Similar basolateral projections have been reported in vitro in rat ATII cells cultured on porous membranes (26, 27). Alternatively, the cytoplasmic projections and irregularly shaped cells observed in mouse ATII cell cultures could be indicative of type I-like differentiation of the cells. In vivo, type II cells are progenitors to type I cells, which are composed of a single nucleated plate joined to several nonnucleated plates by narrow connections (28). The cytoplasmic projections seen in mouse ATII cell cultures (Figure 2C through F) resemble ATII projections that develop in the postnatal period during type I cell differentiation (29). Although differentiation of cultured ATII cells toward a type-I-like phenotype has been reported (21, 24, 30, 31), cytoplasmic projections in contact with neighboring cells have not been previously described in this cell type.
In summary, viable alveolar type II cells can be isolated in high purities and yields from mice. Cultures of these cells contain minimal contamination by Clara cells, mesenchymal cells, or endothelial cells. Cultured ATII cells from mice attach to fibronectin and flatten similarly to cultured ATII cells from rats. However, the cells attach and spread slowly, many are irregularly shaped with numerous cytoplasmic projections, and the majority retain lamellar bodies for at least 48 h after plating. These characteristics indicate that murine ATII cultures have distinct differences from rat ATII cell cultures, and that the cultures may provide useful systems for the study of ATII cell biology. This new method also provides a vehicle for studying the detailed role of ATII cells in well-described in vivo murine lung injury models and, with the availability of many strains of genetically defined mice and transgenic technology, the method may provide powerful new systems for the investigation of the roles of specific genes in distal lung pathobiology.

Acknowledgments: This study was supported by the Defense Nuclear Agency, and N.I.H. grants P42-ES05946 and R01-ES06766-01. Special thanks to Aura Maldonado for ultrastructural preparations and to Victoria Wessells for technical work on Clara cell isolations.

References