DENDRITIC CELLS WITH ANTIGEN-PRESENTING CAPABILITY RESIDE IN AIRWAY EPITHELIUM, LUNG PARENCHYMA, AND VISCERAL PLEURA

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A number of distinct cell types, including dendritic cells (DC) (1), macrophages (2), Langerhans' cells (LC) (3), resting or activated B cells (4), endothelial cells (5, 6), and fibroblasts (7) are capable of presenting antigen to and acting as accessory cells for the activation of antigen-specific T lymphocytes. Although these cell types differ markedly in their effectiveness as APC, they all share certain common features, including the expression of MHC class II antigens, the ability to process antigen, and the ability to secrete IL-1. Within the normal bronchopulmonary system, only alveolar macrophages (AM) express MHC class II antigens (8). Although AM play a central role in host defense, considerable controversy exists with regard to their ability to present antigen, with disparate results being obtained in different species (9–14). Furthermore, AM are primarily found in the alveoli, and one would expect the large areas of mucosa of the tracheobronchial tree to also be sites of antigen presentation. To evaluate this possibility, we have examined preparations of human and mouse bronchial epithelium for the presence of additional cells with antigen-presenting capability. Described herein are the distribution, light and electron microscopic characteristics, and function of a type of bone marrow-derived, MHC class II-bearing DC found widely within the bronchopulmonary tree as well as in the pleura. It is likely that these cells play a central role in antigen presentation in the lung.

Materials and Methods

Human Tissues. Specimens were obtained from the lower trachea and mainstem bronchi from 15 patients during routine diagnostic bronchoscopy. The final diagnoses in these patients were diverse, and included bleeding of unknown cause, carcinoma, tuberculosis, and idiopathic pulmonary fibrosis. The tissues were immediately snap-frozen in liquid nitrogen and kept at −70°C before use. Peripheral lung tissues, including visceral pleura, were obtained during the course of surgery for carcinoma of the lung. Only

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Abbreviations used in this paper: AM, alveolar macrophage; DC, dendritic cell; LC, Langerhans' cell; PLP, periodate-lysine-paraformaldehyde.

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samples containing normal tissue free of carcinoma were used for this study. Some of these samples were immediately fixed for 4 h at 4°C in periodate-lysine-paraformaldehyde solution (PLP) and then washed at 4°C with PBS containing 5% sucrose for the staining procedures. Other samples were frozen in liquid nitrogen and stored at -70°C until used.

**Mouse Tissues.** BALB/c mice were obtained from the Division of Research Services, NIH. After exsanguination, whole lungs were excised and fixed by infusion of PLP through the trachea to obtain samples with well-expanded parenchyma. The samples were then frozen and stored at -70°C. Tracheal epithelium was obtained as described previously (15). Each trachea was removed in toto; the external surface was cleaned of fat and connective tissue, and the specimen was then placed in RPMI 1640 medium. Specimens obtained in this manner were free of contamination by peripheral blood and alveolar macrophages. The trachea was opened along the membranous septum and cut with a scalpel into sections measuring ~2 × 4 mm. These samples were immersed in a plastic culture dish in 5 ml of a 20 mM EDTA solution, pH 7.4, and incubated at 37°C in an atmosphere with 95% O₂ and 5% CO₂. After 2 h in EDTA, the epithelium was lifted from the underlying tissue with curved forceps. The basement membrane appeared intact on histological analysis. Epithelial sheets to be stained were washed three times in PBS containing 0.02% sodium azide and 1% BSA. Tissues for the APC experiments were washed in supplemented DMEM with 10% FCS. The tracheal sheets were disrupted by gentle pipetting to obtain single-cell suspensions.

Enriched populations of mouse alveolar macrophages were obtained by bronchoalveolar lavage with PBS. This cell population was used without further purification and contained 90% macrophages by morphologic criteria.

**Antibodies.** The OKT6, OKT3, and OKM1 antibodies were purchased from Ortho Diagnostic Systems, Raritan, NJ. Leu-1, Leu-2, Leu-3, FITC-conjugated anti-HLA-DR, anti-Ia⁴, anti-Thy-1.2 FITC-conjugates and anti-IgM FITC-conjugates were from Becton Dickinson Monoclonal Center, Mountain View, CA. Anti-IgA, -IgG, -IgM, Dako pan-B, and Dako C3bR were from Dako Corporation, Santa Barbara, CA. Anti-Ia M5-114 (16), anti-Ia M1/70 (18) mAb were obtained from American Type Culture Collection, Rockville, MD. The anti-Fc-R 24G2 mAb, originally prepared by Unkeless (19), was kindly provided by Dr. D. Siegal (NCI, NIH). Some of the mAb were used as FITC-conjugates in direct staining procedures. For indirect staining procedures, the mAb were followed either by a second FITC- or rhodamine-conjugated F(ab')₂ goat anti–mouse IgG and IgM antibody (Grub Antibodies, Scandia, Vienna, Austria, or Tago, Inc., Burlingame, CA) or by FITC- or rhodamine-conjugated goat anti–rat IgG (Tago, Inc., or Cappel Laboratories, Cochranville, PA). For immunoelectron microscopy, goat F(ab')₂ anti–mouse IgG conjugated with peroxidase (Tago, Inc.) was used as the second antibody.

**Staining Procedures.** For light microscopy, the frozen tissues (human bronchial mucosa, human and mouse lung) were cut with a cryostat at -20°C to a thickness of 4 µm. After being air-dried on the slide, sections were fixed in cold acetone for 10 min and washed in PBS. The sections were incubated with the first antibody in the proper dilution in PBS-BSA-azide for 20 min, washed, and then treated with the second antibody. When the Mac-1 and 24G2 mAb were used, a three-step method was employed (20). The second antibody was a polyclonal mouse anti–rat Ig (Jackson Immunoresearch Laboratories, Avondale, PA), and the third antibody was a FITC- or rhodamine-conjugated goat anti–mouse Ig.

In experiments using double-staining, the second antibody was rhodamine-labeled. After being air-dried on the slide, sections were fixed in cold acetone for 10 min and washed in PBS. The sections were incubated with the first antibody in the proper dilution in PBS-BSA-azide for 20 min, washed, and then treated with the second antibody. When the Mac-1 and 24G2 mAb were used, a three-step method was employed (20). The second antibody was a polyclonal mouse anti–rat Ig (Jackson Immunoresearch Laboratories, Avondale, PA), and the third antibody was a FITC- or rhodamine-conjugated goat anti–mouse Ig.

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For immunoelectron microscopy, the human and mouse lung tissues were prefixed with PLP and washed three times with PBS-sucrose. Frozen sections prefixed with PLP were cut to a thickness of 20–50 μm. Sections were then incubated overnight at 4°C in the first antibody, washed, and incubated for an additional 24 h at 4°C with a peroxidase-labeled second antibody. The tissues were then fixed in 1.25% glutaraldehyde in PBS, preincubated in 0.03% 3,3'-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) in 0.05 M Tris-HCl buffer for 30 min at room temperature, reacted with a mixture of 0.01% hydrogen peroxide and 0.05% 3,3'-diaminobenzidine for 10 min, postfixed with 1% osmium tetroxide for 1 h, dehydrated in graded alcohols, and finally embedded in Polybed 812. Ultrathin sections were cut and mounted on copper grids, lightly stained with uranyl acetate, and examined using a JEOL 100B transmission electron microscope.

Cell suspensions from the mouse trachea were similarly processed for electron microscopy after being washed with PBS-BSA-azide and then exposed to the first antibody for 30 min and the second antibody for a final 30-min period. All of the subsequent procedures were done as for the tissue sections.

Cytochemistry for Nonspecific Esterase. Frozen sections of human and mouse lung cut to a thickness of 4 μm were fixed with cold buffered formalin for 30 s and washed with distilled water. These samples were incubated in a mixture of α-naphthyl acetate (N-8505; Sigma Chemical Co.) and Fast Blue BB salt (F-0250; Sigma Chemical Co.) in 0.1 M Tris-maleate buffer, pH 7.7, in a Coplin jar at room temperature for 5 min. After incubation, they were counterstained with 0.1% nuclear fast red and mounted in glycerin-jelly medium.

Assay for Antigen Presentation. An OVA-specific hybridoma, A 2.2.E10, prepared from the draining lymph nodes of OVA-primed BALB/c mice as described previously (21), was used as a responder cell population. Epithelial cells and AM were washed, irradiated with 3,000 rad, and cultured for 48 h with the responder T cell hybridoma (10⁵ cells) with or without antigen in a final volume of 0.2 ml in 96-well culture dishes. After 48 h, 100 μl of the culture supernatant were removed and added to 5 × 10⁵ CTLL cells, an IL-2-dependent T cell line. After 24 h, the degree of stimulation of the CTLL was determined by measuring the incorporation of [³H]thymidine, as described previously (22).

Treatment with Anti-Ia and C. The epithelial cells were incubated for half an hour at room temperature with the rat mAb M5/114. After being washed, a mouse anti-rat κ chain mAb, MAR 18.5 (23), was added to the cells as a facilitating reagent in the presence of nontoxic rabbit C (Cedarlane Laboratories, Hornby, Ontario, Canada) and incubated for 40 min at 37°C. The C-treated cells were added as accessory cells to the culture described above.

Results

Light Microscopy. HLA-DR⁺ (Ia⁺) DC were observed in a rather regular distribution within the epithelium of the large airways of the human lung (Fig. 1 a). These cells were usually found either interspersed perpendicularly between columnar epithelial cells or just above the basal lamina with extending cytoplasmic processes. The cell processes did not usually extend to the bronchial lumen. The population of HLA-DR⁺ DC represented ~1% of all the epithelial cells. In small airways (Fig. 1 b), DC were present between the epithelial cells and appeared to extend toward the lumina of the airways. They were also detected in the vascular walls (not shown), and were found peripherally as far as the alveolar septa (Fig. 1 c) and the visceral pleura (Fig. 1 d). DC in the visceral pleura exhibited extensive cytoplasmic processes arranged parallel to the mesothelial lining. The DC were generally very elongated, measuring 25–40 μm in total length and 5–8 μm in width, with two to three slender cytoplasmic processes extending some distance from the cells. They did not contain vacuoles or ingested particles and were negative for α-naphthyl acetate esterase, which is considered...
Figure 1. Localization of HLA-DR+ DC in normal human lung. (a) Three HLA-DR+ DC are localized in the basal layer of epithelium in a large bronchus. Some dendrites encircle the basal cells and do not reach the bronchial lumen (indirect immunofluorescence, × 400). (b) Several HLA-DR+ DC in the epithelial layer of a small bronchus. Slender cytoplasmic processes extend between the epithelial cells, reaching the lumen. (c) HLA-DR+ DC in interalveolar septa have long cytoplasmic processes. (d) HLA-DR+ DC in the visceral pleura are arranged in parallel with the pleural surface, and show dendrites adjacent to the alveolar epithelial cells. AM are also positive. (Avidin-biotin peroxidase complex method, × 400, used for b, c, and d).

The most reliable cytochemical marker for macrophage identification. On the other hand, the AM were readily identified, appearing as large, rounded cells in alveolar lumina and ovoid in the interstitium, and were strongly positive when stained with nonspecific esterase. AM were HLA-DR+ and sometimes contained ingested particles.

Similar results were obtained when mouse lung tissue was analyzed by immunofluorescence (Fig. 2) or immunoperoxidase staining. The Ia+ DC were found just above or beneath the basement membrane in the bronchial epithelium, with slender, elongated cytoplasmic processes usually arranged parallel to the surface
The Ia+ DC in alveolar walls exhibited elongated dendrites extending towards the alveolar lumina (Fig. 3, b and c). DC in the visceral pleura had cytoplasmic processes that extended parallel to the mesothelial lining (Fig. 3 d), much as seen in the same cells in the human lung. In the cell suspension preparation, ~1% of the cells were Ia+.

Electron Microscopy. Human HLA-DR+ DC in the bronchial epithelium (Fig. 4) and in alveolar walls (Fig. 5 a) exhibited elongated cytoplasmic processes, a euchromatic oval nucleus, mitochondria, endoplasmic reticulum, DR+ coated vesicles, and 80–100-Å-thick microfilaments (Fig. 5 b). The AM had small filopodia and contained numerous lysosomes, some of which had concentric lamellae (Fig. 5 c). The plasma membranes of the AM showed a continuously positive reaction to HLA-DR. No Birbeck granules were found in the mouse or human lung DC.

In the murine tracheal cell suspensions, the Ia+ DC appeared as elongated cells (Fig. 6 a), measuring ~20–30 μm in total length. Each cell had two or three thin dendritic processes measuring 0.1–0.6 μm in width and ~6 μm in length, but no microvillous projections were apparent on the cell surfaces (Fig. 6 b). The DC had oval, euchromatic nuclei and a rather lucent cytoplasm. While the cytoplasmic organelles were not prominent, they had mitochondria, a rather well-developed Golgi apparatus and endoplasmic reticulum, several coated vesicles, and relatively few microfilaments. The cytoplasmic processes contained mitochondria, microfilaments, and Ia+ coated vesicles.

To further characterize the cell surface antigens, we used commercially available mAb (Table I). In the human tracheal tissue, the DC were positive for HLA-DR and the T200 antigen, initially detected individually and later also by double staining (Fig. 7, a and b). These cells were negative for the OKT6 antigen, seen typically in LC. Double staining revealed no T cell markers (OKT3, Leu-1, Leu-2, or Leu-3) or B cell markers (surface Ig and pan-B from Dako, Inc.) on the HLA-DR+ DC. Furthermore, we detected no receptors for C3b or C3bi (OKM1).
FIGURE 3. Localization of Ia+ DC in the normal mouse lung (avidin-biotin peroxidase complex method, all × 400). (a) Ia+ DC lie just above or beneath the basal lamina of the bronchus. Cytoplasmic processes extend to more than three to four basal cells. (b and c) Ia+ DC, as seen using bright field (b) and Nomarski differential interference contrast (c) microscopy, have slender, elongated dendrites towards the alveolar lamina. (d) Ia+ DC in the visceral pleura show long dendrites running parallel to the pleural surface.
FIGURE 4. Immunoelectron microscopy of HLA-DR⁺ cells and dendrites in the epithelium of a small bronchus, showing oval nucleus, HLA-DR⁺ coated vesicles, endoplasmic reticulum, and short cytoplasmic processes lying over the basal cells, but no Birbeck granules (indirect immunoperoxidase method, × 15,000).

The mouse DC also were positive for Ia and the T200 antigen (Fig. 8, a and b). In double-staining experiments of cell suspension preparations, we found that DC were positive for the Fc-IgG (Fig. 8, c and d) and the C3bi receptor (Fig. 8, e and f). Since no staining by anti-Thy-1.2 or anti-IgM was detected, it appears that this cell population was not of T or B cell origin.

Antigen-presenting Capability of Murine Tracheal DC. Because the Ia⁺ population present in the bronchial epithelium resembles splenic DC and the epidermal LC population in many respects, we compared the ability of the tracheal epithelium suspension and AM to present OVA to an OVA-specific T cell hybridoma (Table II). Both cell populations functioned efficiently as APC. However, since the tracheal epithelial population contained only 1% Ia⁺ cells, while 10–30% of the AM were Ia⁺, it appeared that the epithelial DC were highly efficient APC.
Antigen presentation of AM might be due in part to contaminating DC. To confirm that the only APC in the cell suspension were the Ia+ DC, epithelial cells were treated with anti-Ia and C or C alone, and then tested for antigen-presenting
function (Table III). Anti-Ia and C treatment resulted in marked diminution of this function, as reflected in reduced T cell activation. This result strongly suggests that in the trachea, one role of Ia+ DC in the epithelial cell population is antigen presentation.
**Table I**

| mAb           | Human | mAb           | Mouse |
|---------------|-------|---------------|-------|
| HLA-DR        | +     | Ia            | +     |
| T200          | +     | T200          | +     |
| OKT6          | -     | Fc-IgG        | +     |
| OKT3          | -     | Mac-1         | +     |
| Leu-1, -2, -3 | -     | Thy-1,2       | -     |
| a IgA, IgG, IgM | -   | a IgM         | -     |
| B1            | -     |               |       |
| OKM1          | -     |               |       |
| C3b           | -     |               |       |

mAb were used to stain sections or cell suspensions. Determinations were done on 15 separate patients, and each result was repeated at least four times in the mouse system. Double staining was done on three separate experiments (using HLA-DR or anti-Ia and another mAb).

**Figure 7.** Two views of the same field, showing double immunofluorescent staining of normal human bronchial epithelium (× 400). The epithelial layer is at the top; elastic fibers (autofluorescent) are at lower right. a shows one cell that is HLA-DR⁺ positive; b shows two T200⁺ cells, one of which is the same cell shown in a.

**Discussion**

We have shown in this report that the DC population in both human and murine bronchial epithelium, as well as in the alveoli and visceral pleura, has many features in common with the DC in lymphoid organs and with epidermal LC. All of these populations express easily detectable MHC class II antigens (1, 24, 25) and all appear to be bone marrow–derived, as indicated by the expression
of the T200 antigen; none of these populations show reactivity when stained for
nonspecific esterase. Some minor differences between the DC populations were
noted. In humans, the LC express both the Fc-IgG-R and the C3b-R (26). We
have not yet examined human pulmonary DC for the expression of the Fc-IgG-
R; however, we were unable to detect the C3b- or C3bi-R. The majority of the
pulmonary DC in the mouse expressed C3bi- and Fc-IgG-R, as has been observed
TABLE II
Antigen Presentation by Murine Alveolar Macrophages and DC

| Cell number* | [3H]Thymidine uptake* |
|--------------|-----------------------|
|              | Alveolar macrophages   | Epithelial cells |
| 5 × 10⁴ (no OVA) | 3,000                 | 3,100 |
| 5 × 10⁴       | 34,800                 | 29,200 |
| 3 × 10⁴       | 22,500                 | 25,800 |
| 10⁴           | 15,100                 | 10,000 |
| 5 × 10³       | ND                     | 8,800 |
| 10³           | ND                     | 5,900 |

10⁵ T hybridoma cells were cultured for 48 h with the specified number of either alveolar macrophages or epithelial cells containing DC, with or without antigen. Supernatants from these cultures were assayed at a 1:2 dilution on CTLL cells for 42 h. For the last 18 h, the cells were pulsed with [3H]thymidine. OVA concentration, 100 μg/ml.

* Cell number reflects ~90% alveolar macrophages by morphological criteria; 10-30% were la. Epithelial cells contained ~1% DC.

† Mean rounded to nearest hundred.

TABLE III
Ablation of Antigen-presenting Function by Treatment with Anti-la and C

| Treatment     | OVA (100 μg/ml) | [3H]Thymidine uptake* | Inhibition (%) |
|---------------|-----------------|-----------------------|----------------|
|              |                 | 900                   |                |
|              |                 | + 5,300               |                |
| C            |                 | + 7,000               | 0              |
| Anti-la + C  |                 | + 1,700               | 87             |

3 × 10⁴ bronchial epithelial cells were either untreated or treated with MAR 18.5 and C alone, or with anti-la, MAR 18.5, and C, and then assayed as described for APC.

* Mean rounded to nearest hundred.

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with murine epidermal LC (20), while splenic DC express only the C3bi-R (personal communication from G. Schuler). LC are the only DC population having the typical cytoplasmic Birbeck granule. The pulmonary DC, like their counterparts in lymphoid organs and skin, appear to be very effective APC. Unfortunately, we could perform only a limited number of studies because of the low number of DC obtained (10⁴ Ia⁺ cells from 20 mouse tracheas). Nevertheless, impressive activation of an antigen-specific T cell hybridoma was observed. It will be important in future studies to examine the accessory cell function of pulmonary DC in other assays such as mitogen responses, cytotoxic lymphocyte induction, and as stimulator cells of the syngeneic and allogeneic MLR. It is conceivable that important differences may exist in the functional capacity of the different DC populations in stimulating different functional subpopulations of T cells. Because of the low cell yield from our preparations of tracheas, considerable effort was made to prevent contamination of the tracheal cells. Since microscopic examination revealed no erythrocytes, monocytes, or neutrophils in the preparations, it is unlikely that there was any contamination
with peripheral blood cells. Furthermore, no AM were observed, thus excluding the possibility of contamination from the lower respiratory tract. A few lymphocytes were present in the DC population and likely represented lymphocytes that are normally present within the bronchial epithelium (27).

It is quite surprising that a DC population has never previously been described in bronchial epithelium. It has been reported that no LC with typical Birbeck granules were apparent in normal human (28) or rat tracheal epithelium, but such cells have been detected in tracheal tissue in vitamin A deficiency (29), and in alveolar tissue in certain disease states (28). Although the expression of MHC class II antigens by nonlymphoid tissues has been extensively studied, bronchial epithelium has been described as either diffusely positive (8, 30) or completely negative (31, 32). In tissue samples from 3 of our 15 patients, anti-class II reagents produced diffuse staining; however, in the epithelial cells from the other 12 patients, the test results were negative. It is possible that chronic environmental or microbiologic factors leads to class II antigen expression by pulmonary tissues in a manner similar to that seen in keratinocytes in certain diseases (33).

At present, we can only speculate about the physiologic importance of the pulmonary DC. As the pulmonary DC are the only MHC class II antigen–positive cells in the tracheobronchial epithelium, and as they have antigen-presenting capability, it seems reasonable to propose that they play a role in the afferent limb of the immune response, analogous to that proposed for LC in the epidermis (3). Stein-Streilein has recently shown (34) that intratracheal, but not oral or intravenous, application of soluble hapten results in a contact hypersensitivity response when local challenge is applied to the skin. In man, sensitization via the airways may also lead to subsequent acute or chronic inflammatory response in the bronchi and lung, or may even lead to systemic reactivity.

The relationship of the DC in the periphery of the lung to AM must be clarified. In man, purified AM have been reported to be devoid of antigen-presenting function (9), and it is possible that the main role of AM is to degrade foreign antigens, and thereby prevent rather than induce an immune response. As considerable controversy exists in the literature in regard to the antigen-presenting function in rodent AM (10–14), it is possible that such activity observed in some studies was in fact mediated by contaminating DC.

The existence of an MHC class II antigen population of pulmonary DC may have great importance in furthering our understanding of the immunopathology of rejection of lung grafts. It is likely that DC within the graft are a major source of the stimulating alloantigen responsible for graft rejection. Depletion of DC, at least in some animal models (35), leads to a prolongation of graft survival. The pulmonary changes that occur after heart-lung transplantation include obliterative bronchiolitis and significant pleural fibrosis (36). It is possible that donor pulmonary DC contributed to this inflammatory response. Airway obstruction has also been reported in bone marrow transplant recipients (37), and may be a general manifestation of graft-vs-host disease in the lung. Again, the role of pulmonary DC in such a response remains to be determined.
Summary

In this study, we identified a population of dendritic cells (DC) that exists throughout human and mouse pulmonary tissues, including the trachea, bronchi, alveoli, and visceral pleura. In human tissue, these DC were shown to be positive for HLA-DR and T200 antigens. In the mouse, the DC expressed not only Ia and the T200 antigen, but also Fc-IgG and C3bi receptors. Unlike alveolar macrophages, the DC were negative for nonspecific esterase staining and shared ultrastructural similarities with the DC described by Steinman (1), and with Langerhans' cells, even though they did not contain Birbeck granules. We were able to demonstrate that mouse pulmonary DC function in antigen presentation, as observed with the other DC. Thus, the respiratory tract contains DC that are capable of functioning in antigen presentation and that may be important in pulmonary immune responses.

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