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Distribution of Ia antigens and T lymphocyte subpopulations in rat lungs

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SUMMARY

In order to examine the mechanisms of specific immunity in the lung, the distribution of Ia antigens and T lymphocyte populations was determined using immunoperoxidase-staining of cryostat sections of lungs from specific pathogen-free rats. BALT was found to be divided into three regions of lymphoid tissue. The central region was primarily composed of B cells, and was surrounded by a peripheral region of T cells (MRC OX-19$^+$) which included both T helper (W3/25$^+$) and T suppressor/cytotoxic (MRC OX-8$^+$) cells. The subepithelial region contained a dense network of W3/25$^+$, non-T cells. A majority of BALT cells, including the lymphoepithelial cells, were Ia$^+$. The alveolar walls were found to contain numerous Ia$^+$ dendritic-shaped cells. Alveolar macrophages found in sections, as well as those collected using bronchoalveolar lavage, were Ia$^-$ and W3/25$^-$. Mechanisms for the induction of immunity within both BALT and the alveolar region are proposed.

INTRODUCTION

B and T lymphocytes collaborate in the induction and control of immune responses. T lymphocytes can be grouped into two regulatory subpopulations that either positively or negatively influence the production of an immune response. In the rat, MRC OX-8 and W3/25 monoclonal antibodies are used to identify T suppressor/cytotoxic cells and T helper cells, respectively (Brideau *et al.*, 1980; White *et al.*, 1978).

The major histocompatibility complex (MHC) encodes for cell surface molecules which control the interaction between these lymphoid cells. One group of MHC molecules of major interest is the class II or Ia antigens, because the induction of an antigen-specific immune response requires that the immunizing antigen be presented to a T cell in association with Ia antigens on the surface of an accessory cell. Ia antigens are found on B cells, some T cells, accessory cells and some epithelial cells.

The lung appears to be protected by two overlapping immune systems, the mucosal immune system (BALT) and systemic immune system (bronchoalveolar immunity) (Clancy & Bienenstock, 1974; Beacham & Daniele, 1982). Thus, in order to examine the mechanisms of specific immunity in the lung, the location of lymphocytes and their subpopulations needs to be determined prior to antigenic stimulation. Also, the distribution of Ia antigen must be examined due to its requirement in antigen presentation. In this study, the distribution of Ia antigens and T-lymphocyte populations was determined in cryostat sections of lungs in fetal, neonatal, juvenile and adult specific pathogen-free rats. These animals were known to be free of infection by *Mycoplasma pulmonis*, a common respiratory pathogen in laboratory rats that can greatly change the composition of lung lymphocytes in infected rats (Davis *et al.*, 1982). Also, bronchoalveolar lavage cells were examined for the presence of Ia antigens on their surface. From this study, a model for the structural organization of BALT is presented. Also, mechanisms for the induction of immunity within both BALT and the alveolar region, in terms of the structural organization, are proposed.

Abbreviations: BALT, bronchial-associated lymphoid tissue; MHC, major histocompatibility complex; PBS, phosphate-buffered saline.

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MATERIALS AND METHODS

Animals

F344/Ld rats were from a specific pathogen-free colony, maintained in Trexler plastic film isolators (Cassell & Davis, 1978; Lindsey *et al.*, 1971) at the University of Alabama, Birmingham, and shown, using ELISA serology and culture (Cassell *et al.*, 1981; Davis & Cassell, 1982), to be free of mycoplasmas, murine viruses and bacterial pathogens. The majority of the experiments were performed with 10-week-old male rats (adults). Four-week-old males (juvenile), newborn (less than 1 day old) and fetuses (about 17–18 days gestation) were used where noted. Three rats from each of the age groups were used for lung sections.

Antibodies

The monoclonal antibodies used in this study were gifts from Drs A. N. Barclay and A. F. Williams (MRC Cellular Immu-

Table 1. Monoclonal antibodies used in experiments

Antibody	Antigen or tissue specificity
MRC OX-1	Leucocyte-common antigen*
MRC OX-6	Ia antigen (rat homologue to mouse I-A)†
MRC OX-8	T-suppressor/cytotoxic cells, NK cells‡
MRC OX-17	Ia antigen (rat homologue to mouse I-E)†
MRC OX-19	T cells§
MRC OX-21	Human C3b inactivator¶
W3/25	T-helper cells, macrophages, dendritic cells**

* Sunderland, McMaster & Williams (1979).
† Fukumoto, McMaster & Williams (1982).
‡ Brideau *et al.* (1980); Woda *et al.* (1984).
§ Dallman, Thomas & Green (1984).
¶ Hsuing *et al.* (1982).
** White *et al.* (1978); Barclay (1981); A. N. Barclay (personal communication).

nology, Sir William Dunn School of Pathology, Oxford, U.K.), and are briefly described in Table 1. Rabbit anti-rat $F(ab')_2$ and rabbit $F(ab')_2$ anti-mouse IgG were prepared (Williams, Galfre & Milstein, 1977; Jensenius & Williams, 1979). Rabbit $F(ab')_2$ anti-mouse IgG and goat $F(ab')_2$ anti-rabbit IgG adsorbed with rat immunoglobulin and conjugated with horseradish peroxidase as previously described (Nakane & Kawaoi, 1974).

Preparation of tissues

Four- and 10-week-old rats were sedated using a combination of ketamine hydrochloride (Ketaject: Bristol Laboratories, Syracuse, NY) and pentobarbital sodium (The Butler Co., Columbus, OH). The lungs were removed and intratracheally perfused with 85% (v/v) solution of OCT (optimum cutting temperature) compound (Tissue-tek II: Lab-Tek Division, Miles Laboratories Inc., Naperville, IL). Individual lobes were separated, embedded and frozen in OCT compound; 4-μm cryostat sections were cut. Newborn rats were sedated by hypothermia (4°), and their entire lungs were removed, embedded, frozen and sectioned. Fetuses were decapitated, embedded whole and sectioned. All sections were air-dried and stored at 4° with desiccant until ready for use.

Preparation of bronchoalveolar lavage cells

In order to prevent contamination by blood lymphocytes and monocytes, the lungs were first perfused as follows. The inferior vena cava was clamped, the abdominal aorta was cut, and 20–30 ml of phosphate-buffered saline (PBS) with heparin (5 units/ml) were injected into the right ventricle of the heart until the lungs were completely cleared of blood.

Bronchoalveolar lavage cells were then collected from 10-week-old F344 rats as previously described (Davis *et al.*, 1980). The cells were washed twice and resuspended in RPMI-1640, 10 mM HEPES (*N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulphonic acid; Research Organics, Cleveland, OH), 1% L-glutamine (Gibco, Grand Island, NY) and 5% fetal calf serum (Hyclone, Logan, UT). The cell suspension was either smeared onto glass slides or placed onto acid-cleaned coverslips and incubated for 30 min at 37°. The non-adherent cells were washed off the coverslips. Both slides and coverslips were air-dried and stored at 4° with desiccant until ready for use.

Immunoperoxidase staining

Sections, coverslips and slides were stained as previously described (Barclay, 1981). Briefly, sections and cells were fixed in absolute ethanol (4°), washed with PBS containing 2% bovine serum albumin, incubated with the appropriate monoclonal antibody or with rabbit anti-rat $F(ab')_2$, washed, and incubated with the appropriate peroxidase-conjugated antibody in the presence of 10% rat serum. The location of the peroxidase was revealed by incubation with 3,3'-diaminobenzidine HCl. The slides were lightly counterstained with Harris' haemotoxylin, unless otherwise noted. The slides were photographed using a Leitz Dialux 20 microscope, blue filters and Kodak Technical Pan 2415 black and white film (Eastman Kodak Co., Rochester, NY).

RESULTS

Specificity of staining

Neither MRC OX-21 monoclonal antibody plus peroxidase-conjugated rabbit $F(ab')_2$ anti-mouse IgG nor peroxidase-conjugated goat $F(ab')_2$ anti-rabbit IgG reacted with rat lung tissue. However, minimal endogenous peroxidase activity was associated with red blood cells but did not interfere with the interpretation of the slides.

Distribution of Ia antigens and lymphocyte populations in BALT

Lymphoepithelial cells, as well as cells immediately beneath the lymphoepithelium, reacted intensely with both MRC OX-6 and MRC OX-17 antibodies (Fig. 1c); in contrast, normal bronchoepithelium was consistently negative using these reagents. These monoclonal antibodies react with the rat homologues of mouse I-A and I-E, respectively (Fukumoto, McMaster & Williams, 1982). Using MRC OX-1 antibodies (Sunderland, McMaster & Williams, 1979), both lymphoepithelial and bronchoepithelial cells were negative for leucocyte-common antigen.

Immediately beneath the lymphoepithelium, a dense network of cells reacted strongly with both Ia reagents (Fig. 1c). A few small, heavily stained MRC OX-19$^+$ cells were found immediately beneath the lymphoepithelial surface (Fig. 1a). In contrast to MRC OX-19$^+$ cells, more W3/25$^+$ cells were present near the lymphoepithelium (Fig. 1b). This suggests that a large portion of these cells were Ia$^+$, W3/25$^+$, non-T cells.

Almost all cells within the BALT nodules were MRC OX-1-positive (Fig. 2e). MRC OX-19$^+$ cells were located primarily at the peripheral rim of BALT, and were concentrated on the antiluminal side of the BALT nodules (Fig. 2b). W3/25 and MRC OX-8 antibodies both stained cells at the periphery of the nodules similarly to MRC OX-19 (Fig. 2c, d). However, some W3/25$^+$, dendritic-shaped cells were found in the centronodular area of BALT when counterstain was not used (not shown). Immunoglobulin-positive cells were found primarily in the interior of the nodules, not in MRC OX-19-positive areas (Fig. 2a).

Distribution of Ia antigens and lymphoid cell populations in the alveolar region

Within the alveolar walls, numerous pleomorphic cells stained with anti-Ia reagents (Fig. 3b); however, type 1 and type 2

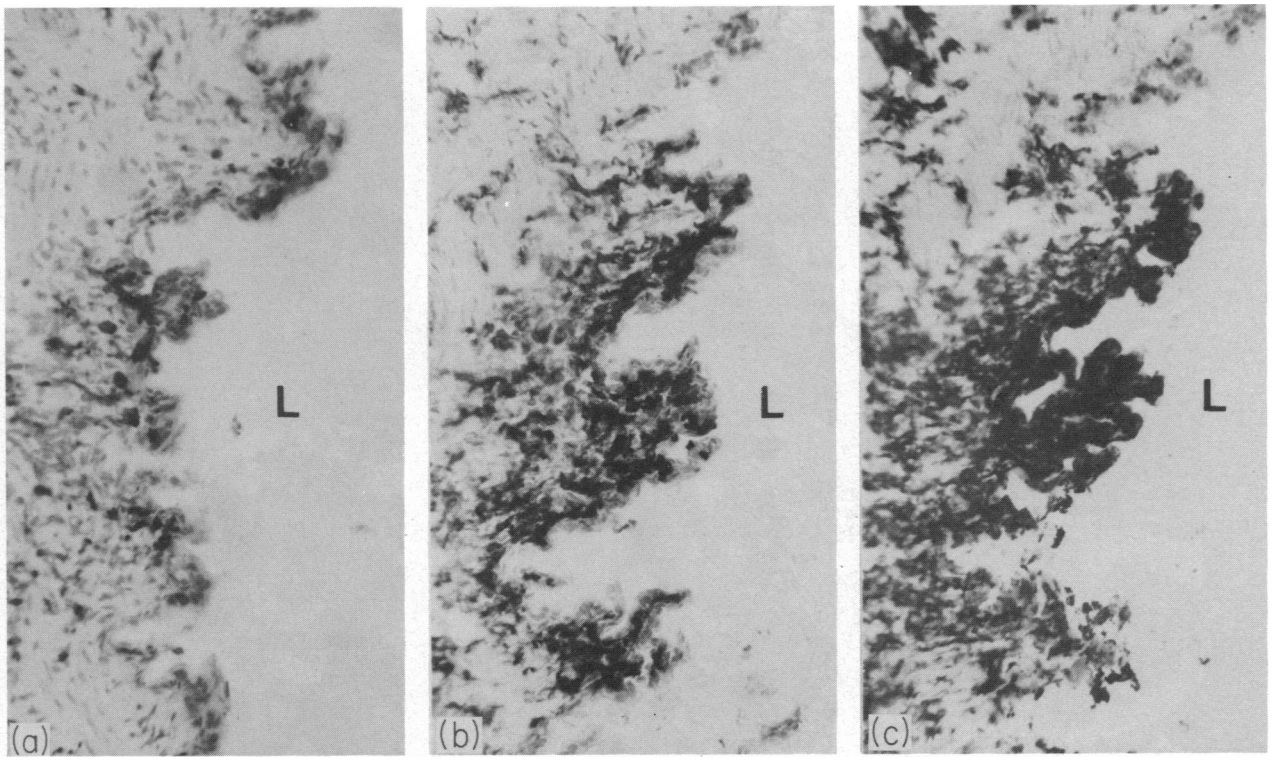

Figure 1. BALT lymphoepithelium. The lymphoepithelium did not display any reactivity to (a) MRC OX-19 and (b) W3/25, but intensely reacted with (c) MRC OX-6. There was a higher proportion of W3/25$^+$ cells than MRC OX-19$^+$ cells immediately below the lymphoepithelium. L, bronchial-lumen. Magnification × 1200.

pneumocytes did not stain with these reagents. Light staining of cells in alveolar walls, morphologically similar to the Ia$^+$ cells, by W3/25 antibody was seen in sections not counterstained. There were no cells that stained with MRC OX-8 antibody in the alveolar walls.

All cells free in the alveolar spaces were MRC OX-1$^+$. In contrast, Ia reagents stained only a few cells in the alveolar spaces. A number of large, foamy cells staining with Ia reagents was found in some sections, but their presence varied between animals. This suggests that a majority of alveolar macrophages were Ia$^-$.

MRC OX-19$^+$ cells occurred infrequently in the alveoli, but W3/13$^+$ cells were present in large numbers. W3/13$^+$ cells had a distribution similar to MRC OX-1$^+$ cells in this region; however, while some cells free in the alveolar spaces were W3/13-negative, they were all MRC OX-1-positive. Along the alveolar walls, round MRC OX-8$^+$ cells were found. No W3/25$^+$ cells were free in the alveoli.

Cells collected by bronchoalveolar lavage were stained with MRC OX-1 antibody. Neither the cell smears nor the adherent cells displayed any reactivity toward W3/25, MRC OX-6 or MRC OX-17 antibodies, thus confirming the lack of Ia and W3/25 antigens on alveolar macrophages as indicated in the lung sections.

Ontogeny of lung lymphoid tissue and the distribution of Ia antigens

Neither fetal (about 18 days gestation) nor neonatal lungs (about 1 day old) contained any lymphoid aggregates; however, both contained a large number of randomly distributed, pleomorphic cells which were Ia$^+$ (Fig. 4) and MRC OX-1$^+$.

By 4 weeks of age, the distribution of lung lymphocytes and Ia antigens was the same as found in adult (10-week-old) rats. Thus, the development of lung lymphoid tissue occurs within the first month after birth.

DISCUSSION

BALT is divided into three anatomic regions (Racz *et al.*, 1977): the region in the centre of BALT (centronodular region); the region surrounding the centronodular region, especially on the anti-luminal side of BALT (peripheral rim); and the region immediately beneath the lymphoepithelium (subepithelial region). In this study, we found that each of these regions was composed of different types of lymphoid cell populations.

The centronodular region was composed of densely packed lymphocytes which were primarily composed of B cells as shown by positive reactivity to anti-rat F(ab)$_2$ antisera. There were a larger number of W3/25$^+$ dendritic-shaped cells than MRC OX-19$^+$ cells in this region, thus W3/25$^+$, MRC OX-19$^-$ dendritic-shaped cells were present in the centronodular region. Since macrophages and dendritic cells have similar phenotypes to these cells (Barclay, 1981; A. N. Barclay, personal communication), the W3/25$^+$, MRC OX-19$^-$ cells may be of the same or similar lineage and presumably perform similar functions, such as accessory cell activity.

The peripheral rim of BALT was composed of loosely associated lymphocytes, some of which were T cells (i.e. MRC OX-19$^+$). The cells in the peripheral rim also reacted with W3/

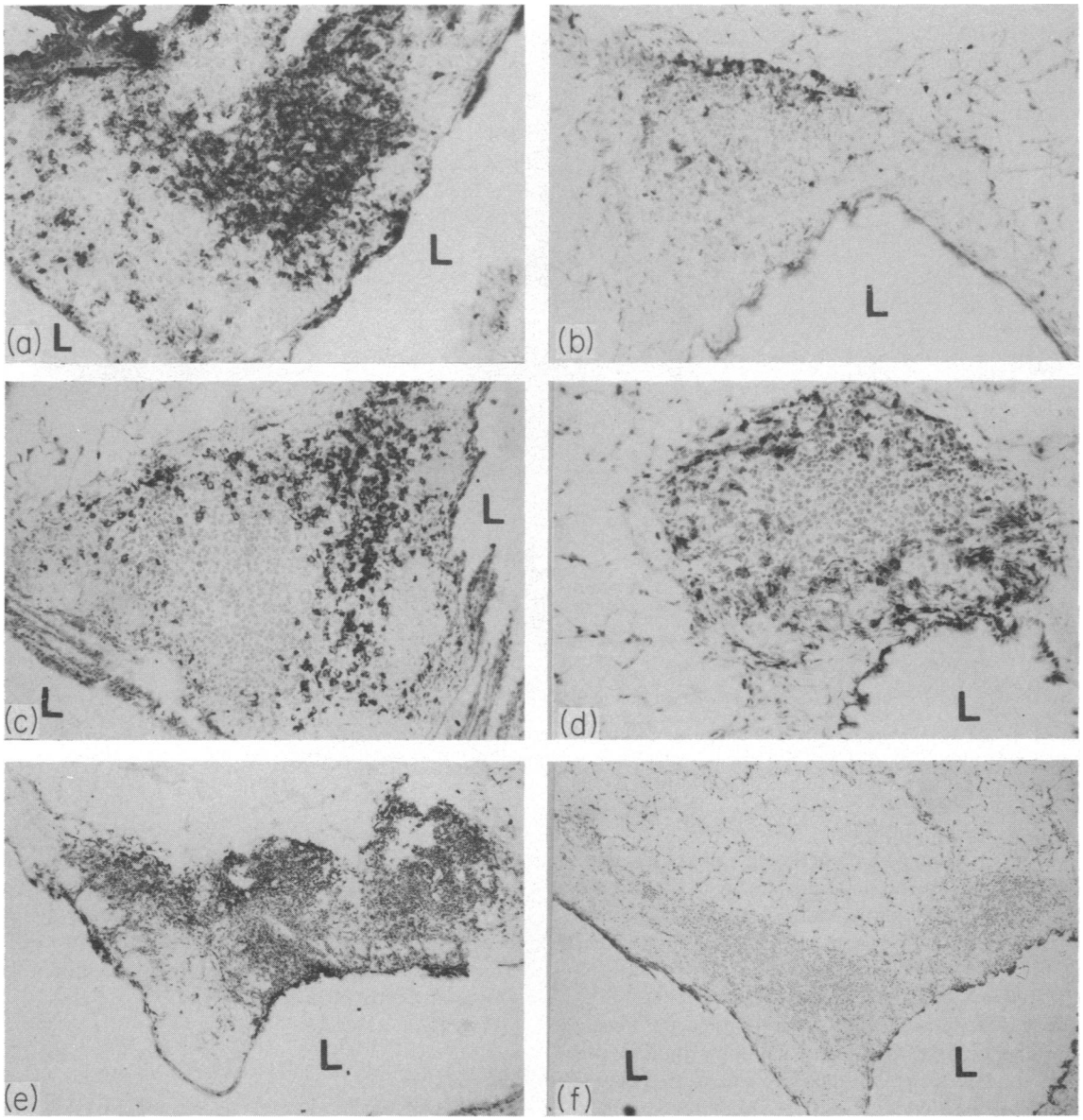

Figure 2. Distribution of lymphocyte subpopulations in BALT. (a) Centronodular region consisted of B cells ($F(ab')_2$-positive); also, the epithelial surface was $F(ab')_2$-positive, presumably due to immunoglobulin present in mucosal secretions (magnification × 600); (b) peripheral region contained T cells (MRC OX-19-positive) especially on the anti-lumenal side (magnification × 560); (c) T cytotoxic/suppressor cells (MRC OX-8-positive) (magnification × 600), and (d) T-helper cells (W3/25-positive) (magnification × 625). The majority of cells in BALT stained with MRC OX-1 (e) (magnification × 325) while no staining was found with MRC OX-21, anti-human C3b inactivator (f) (magnification × 325) L, bronchial-lumen.

25 and MRC OX-8 monoclonal antibodies. Since these monoclonal antibodies bind to T helper and T suppressor/cytotoxic cells, respectively (Brideau *et al.*, 1980; White *et al.*, 1978), the regulatory activities of these T-cell subsets are likely to play a role the immune function(s) of BALT. Also, there appeared to be more MRC OX-8$^+$ cells than MRC OX-19$^+$ cells, indicating the presence of MRC OX-19$^-$, MRC OX-8$^+$ cells in BALT. This cell population may correspond partly to 'null' cells found in isolated lymphocytes from rat lungs (Davis *et al.*, 1982). This cell phenotype (MRC OX-19$^-$ and MRC OX-8$^+$) corresponds to NK cells (Woda, 1984); however, Reynolds, Timonen & Herberman (1981) failed to find this activity in cells isolated from the lungs of rats.

The subepithelial region appeared as a dense network of cells, found in close association with the anti-luminal side of lymphoepithelial cells. Few of these cells reacted with MRC OX-19 monoclonal antibody, and a majority reacted with W3/25 monoclonal antibody. Thus, there was a high number of W3/25$^+$, MRC OX-19$^-$ cells present in the region that are probably related to macrophages and/or dendritic cells for reasons stated above.

The distribution of Ia antigen in the lung was determined using MRC OX-6 and MRC OX-17 monoclonal antibodies which bind to the rat homologues to I-A and I-E antigens, respectively (Fukumoto *et al.* 1982). Since these monoclonal antibodies exhibited identical staining patterns, it is unlikely

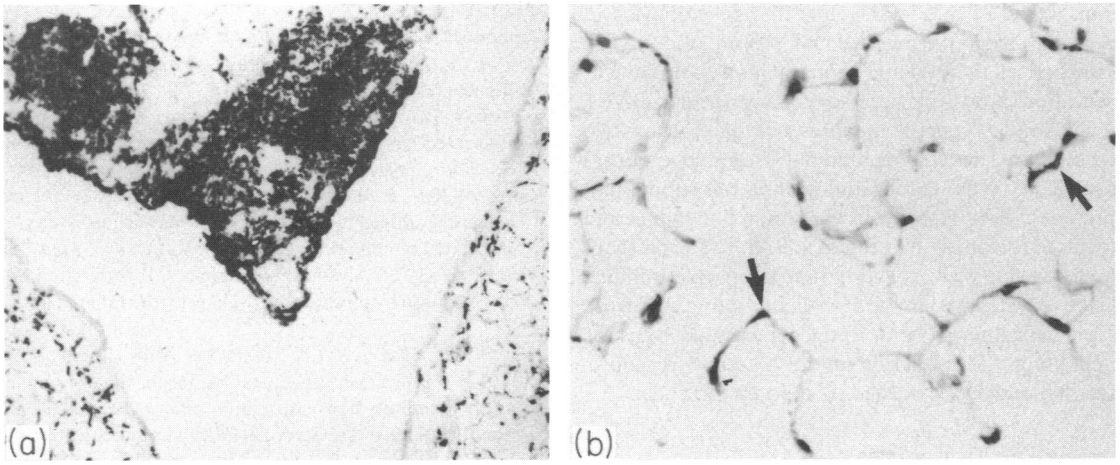

Figure 3. Distribution of Ia in BALT and alveoli. (a) The majority of BALT, including lymphoepithelium, stained with MRC OX-6 antibody (magnification × 350). (b) Pleomorphic cells in the alveolar walls were found to react with anti-Ia reagents (arrows indicate only two of the many Ia^+ cells found in this figure) (magnification × 1100).

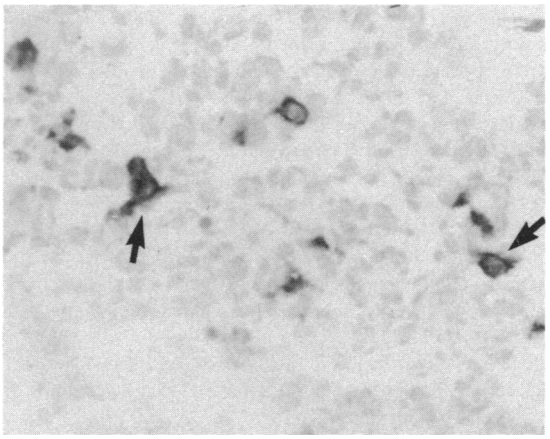

Figure 4. Ia-positive cells in fetal lung. A pleomorphic Ia^+ cell was found in lung at 17–18 days gestation (arrows), as well as in neonatal (less than 1 day old) lung (magnification × 1200).

that there was a cross-reacting non-Ia antigen present in the lung.

A majority of cells in BALT were Ia^+. A previous study has described a similar distribution of Ia^+ cells in BALT (Plesch, 1982); however, in the peripheral rim of BALT, the Ia^+ cells were interdigitating cells, and there was no expression of Ia on the lymphocytes (i.e. T cells). Because of the low number of T cells relative to the rest of the BALT lymphocytes, we were unable to confirm that observation.

Lymphoepithelium is able to transport antigen from the bronchial lumen to the underlying BALT (Gregson, Edmonson & Plesch, 1982; Fournier *et al.*, 1977); however, it is unknown whether lymphoepithelial cells act simply as passive conduits for antigen or whether they process antigen as do macrophages. Our observation that BALT lymphoepithelium, and not bronchoepithelium, was Ia^+ and MRC OX-1^- suggests that they are non-lymphoid cells which may have more than a passive role in immunity. Richman *et al.* (1981) speculated that intestinal lymphoepithelium, which is also Ia^+, may be involved in the presentation of antigen to helper T cells. The presence of Ia antigen, a requirement for accessory cell activity, supports a similar hypothesis for BALT lymphoepithelium. Another hypothesis, proposed by Mayerhofer, Pugh and Barclay (1983) for Ia^+ intestinal lymphoepithelium, states that Ia antigen may play a role in the uptake of antigen from intestinal lumen. Of course, other unknown functions of Ia may be active at this site.

Our observation that the vast majority of rat alveolar macrophages lack Ia antigens on their surface indicates that they were unable to perform as accessory cells in the induction of an immune response. In fact, Holt (1979) has shown that rat alveolar macrophages are suppressive. Also, alveolar macrophages did not express the W3/25 antigen which is present on other rat macrophage populations (Barclay, 1981), confirming the heterogeneity between alveolar macrophages and other macrophage populations (Thomas, Galbraith & MacSween, 1978). Whether alveolar macrophages are able to acquire these antigens after immune stimulation is not known. However, the lack of Ia on alveolar macrophages may be of little consequence, due to the presence of Ia on other cells in the lung and the possible influx of Ia^+ macrophage populations into the lung after immune stimulation.

The presence of Ia^+ cells in alveolar walls was unexpected. These cells were not present in fetal or newborn rat lungs but were present at 4 weeks of age. Thus, Ia^+ alveolar wall cells either develop from a precursor cell in the lung or are acquired via circulation or some other route. In support of the acquisition of these cells via circulation, leucocyte-common antigen (MRC OX-1), which is associated with bone marrow-derived cells, was found on cells with a similar distribution and phenotype as the Ia^+ found in the alveoli.

Although the functions of the Ia^+ alveolar wall cells are unknown, one idea is that they may be able to process and present antigen to lymphocytes migrating from the circulation to either the bronchial tree or to local lymphoid tissues through the pulmonary lymphatics, or processed antigens released by alveolar macrophages may be presented by the Ia^+ alveolar wall cells to these lymphocytes. As with the lymphoepithelium, other unknown functions of Ia may be active at this site.

We also confirmed previous observations (Gregson, Davey & Prentice, 1979) that BALT was not present in rats at birth. By 4 weeks of age, the distribution of lymphoid cells in BALT was

similar to that found in adult rats; thus, the development of BALT appears to occur during the first month of life. The function of the Ia$^+$ cells in fetal and neonatal lungs is unknown.

The observation of possible accessory cells present in BALT and in alveolar walls suggests the presence of at least two anatomically different sites where pulmonary immune responses may be induced. Ia$^+$ cells, B cells and both T-cell subpopulations were present in BALT; thus, all cells required to induce and regulate an immune response are present in BALT. The presence of Ia$^+$ cells in alveolar walls suggests that a separate immune response can be initiated within the alveoli; migrating cells from the circulation may interact with antigen presented by these cells, thus enabling the induction of an immune response. However, further studies are needed to support these ideas.

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