Immunology 1987 **62** 349–356

Localization of T cells, macrophages and dendritic cells in rat respiratory tract tissue: implications for immune function studies

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Accepted for publication 6 July 1987

SUMMARY

Monoclonal antibodies against a range of surface markers were used to localize T cells, macrophages and dendritic cells (DC) in sections of rat trachea and peripheral lung, employing the immunoperoxidase technique. A population of Ia-bearing cells with characteristic DC morphology was identified within the tracheal epithelium, closely associated with the basement membrane, and Ia^+ dendritic processes from these cells penetrated the epithelium reaching the overlying fluid layer. A second DC-like population, also Ia^+ but differing from the airway DC in expression of other markers, was identified within the alveolar septal walls. Both types of DC were intimately associated with populations of pleiomorphic heterogenously staining macrophages. In addition, a large population of T lymphocytes was identified within the alveolar septa; the lymphocytes occurred as single, isolated cells, distributed randomly throughout the lung parenchyma.

INTRODUCTION

The induction and expression of T-cell dependent immunity within the lung is governed by the same overall regulatory mechanisms that apply to the immune system in general. However, the unique features of this organ—in particular, the intimate association of the large tissue vascular bed and the overlying epithelium with the external environment—dictate that local T-cell activation in response to incoming antigens must be tightly controlled, and restricted to encounters with potentially pathogenic antigens.

It is now recognized that active suppression of local T-cell activation, mediated via intrinsic macrophage-mediated (Holt, 1979b; Pennline & Herscowitz, 1981; Holt, 1986) and adaptive T-cell mediated mechanisms (Holt & Sedgwick, 1987), plays an important role in the maintenance of immunological homeostasis in the lung. However, the precise details of how these mechanisms operate in different parts of the respiratory tract, in particular at the level of the initial interaction(s) between resident antigen-presenting cells (APC) and T cells, remain to be elucidated.

It has recently been shown that the major population of APC present in lymph nodes, peritoneal cavity and thoracic duct lymph in the rat, exhibits morphological and phenotypical properties that are consistent with dendritic cells (DC) (Klinkert *et al.*, 1980; Klinkert, La Badie & Bowers, 1982; Mayrhofer, Holt & Papadimitriou, 1986). Additionally, the capacity of these cells to transmit antigen-specific activation signals to T cells *in vitro* was suppressed by the presence of significant numbers of macrophages (Klinkert *et al.*, 1982; Mayrhofer *et al.*, 1986).

In relation to rat lung, we have recently demonstrated class II MHC antigen-bearing (Ia^+) DC in collagenase extracts of lung slices, which exhibit strong APC activity *in vitro* providing that endogenous macrophages are first removed (Holt *et al.*, 1985a; Holt, Schon-Hegrad & Oliver, 1987). This suggests that T-cell activation in the lung *in vivo* may also be controlled by interactions between DC and macrophage populations. In order to gain some understanding of how these processes occur *in vivo*, it is necessary to obtain information on the spatial relationship between the relevant cell populations within particular micro-environments in lung tissue, during the steady state. Accordingly, in the study below we have undertaken a detailed examination of the distribution of macrophages, DC-like cells and T cells within rat lung tissue, employing a panel of monoclonal antibodies directed against a range of cell-surface markers.

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MATERIALS AND METHODS

Animals

Young adult SPF rats of the WAG and BN strains were obtained from the Animal Resource Centre, Murdoch University. Comparable results were obtained with tissue from both strains, and they are not distinguished in the text.

Cell preparation

Free alveolar cells were collected by repeated endobronchial lavage, as detailed in Holt (1979a). Mononuclear cells were

extracted from the parenchyma of peripheral lung samples via collagenase digestion, after initial exhaustive perfusion of the tissue via the airways and blood vessels to remove non-parenchymal cells (Holt *et al.*, 1985b). Tracheal tissue cells were prepared by a modification of the method of Sertl *et al.* (1986). Briefly, individual tracheas were opened with scissors, and incubated in collagenase/DNAse for 20 min, after which the epithelium was scraped from the underlying tissue with fine forceps. The detached tissue was digested for a further 60 min, washed, and sieved repeatedly to remove tissue fragments and debris, yielding a single cell suspension.

Antibodies

The monoclonal antibodies (MoAbs) OX-6, OX-8, OX-12, OX-19, OX-41, OX-42, OX-43, OX-52, W3/13 and W3/25, which were developed in the MRC Cellular Immunology Unit, Oxford, U.K., were kindly supplied gratis from that laboratory by Professor Alan Williams, Dr Don Mason, Dr Ann Robinson and Dr Jon Sedgwick. The ED series of MoAb were kindly provided from their laboratory of origin, by Dr Christine Dijkstra, Department of Histology, Free University, Amsterdam, The Netherlands. Biotinylated rabbit anti-mouse IgG and straptavidin–horseradish peroxidase (HRP) conjugates were purchased from Amersham, Sydney, Australia.

Staining procedures

For fresh frozen sections, lengths of trachea were initially filled with OCT freezing medium (Tissue-Tek II, Miles Laboratories, Elkhart, IN), and embedded by freezing in OCT using liquid nitrogen. Lungs were initially filled with OCT via the trachea, and 5-mm cubes excised and embedded as above. Five micrometre longitudinal or transverse sections were cut, respectively, from trachea and lung using a Bright cryostat, air dried at room temperature for 1 hr, fixed in absolute ethanol at 4° for 10 min, and rehydrated through 70% ethanol to Dulbeccos PBS, before incubation with MoAbs.

Immunoperoxidase detection of antigens was by an indirect technique detailed in Barclay (1981). A mouse MoAb against a human lymphocyte-surface marker was used as a negative control. Incubation of tissue sections with MoAbs was performed at 4° for 1 h, followed sequentially by biotinylated rabbit anti-mouse IgG, and the streptavidin–HRP conjugate to detect bound mouse antibodies. After washing, enzyme-linked antibody was revealed by reacting with 3,3′-diaminobenzidine and hydrogen peroxide for 10 min at room temperature. The sections were then lightly counterstained with haematoxylin.

For detection of antigens on alveolar and tracheal digest cells in suspension, cytocentrifuge preparations or cells which had been allowed to settle onto Alcian blue-treated slides were initially air dried, ethanol fixed and then rehydrated as above, prior to immunoperoxidase staining.

RESULTS

Cell yields from lung tissue

Lung tissue samples from a large series of 150-g adult rats yielded an average of $2{\cdot}3 \times 10^6$ lavage cells, and subsequent collagenase digestion of sliced lung produced a further 1×10^8 mononuclear cells, as reported earlier (Holt *et al.*, 1985b). Individual tracheas averaged $2{\cdot}5 \times 10^5$ cells. Samples of these cells were stained with a panel of MoAb, as shown in Table 1.

Table 1. Immunoperoxidase staining of cell populations from rat respiratory tract tissue

MoAb	Antigen and/or cell specificity	% positive cells in: Lavage fluid	Tracheal digest	Lung digest
OX-6*	Ia; macrophages, B cells, DC	0	28	19 (14)
OX-8*	CD8-T cells, NK cells	3 (3)	ND	18
OX-12*	Kappa chain; macrophages, B cells	ND	ND	52
OX-19/52*†	All T cells	2 (2)	11	35
OX-41‡	Macrophages	95 (98)	20	21
OX-42‡	iC3b receptor; macrophages, DC	22 (19)	18	26 (55)
OX-43§	Macrophages	ND	ND	21
W3/13*	T cells, Plasma cells	ND	ND	37
W3/25‡	CD4-T cells, Macrophages, DC	0 (0)	ND	33 (11)
ED1¶	Macrophages, DC	95 (97)	7	28 (47)
ED2¶	Macrophages, some DC	0	9	5 (18)
ED3¶	Macrophages, some DC	0	ND	0

Data shown for each MoAb were derived from observation of 300–500 cells. Figures for background controls (endogenous peroxidase) were $\leq 2\%$. Figures in parentheses refer to frequencies in macrophage-enriched cell populations, prepared via adherence to glass coverslips during 2-hr incubation at 37° in RPMI plus 10% fetal calf serum, followed by vigorous washing to remove non-adherent cells.

* Reviewed in Mason *et al.* (1983).

† Robinson, Puklavec & Mason (1986a); a mixture of the two MoAb was employed, as OX-19 staining was relatively faint.

‡ Robinson *et al.* (1986b).

§ Robinson, White & Mason (1986c).

¶ Dijkstra *et al.* (1985); Van Der Brugge-Gamelkoorn, Dijkstra & Sminia (1985).

Table 2. Distribution and relative frequency of cells in rat lung tissues stained with a panel of MoAb

	Airways			Peripheral lung				
MoAb	Tracheal epithelium	Tracheal mucosa	BALT	Adherent to alveolar surface	Within alveolar septa	Pleura	Peribronchial and/or perivascular	Alveolar spaces*
OX-6	++	++++	++++	+	++++	++	+++	−
OX-8	±	±	++	−	+	±	±	±
OX-19/52	±	±	++++	−	++	±	+	±
OX-41	±	±	+	±	±	+	−	++++
OX-42	±	+	±	±	++	++	±	+
W3/25	+	++	++	−	++	+	++	−
ED1	+	+	+	±	+	±	+	++++
ED2	−	±	−	−	+	+	++	−

For purposes of comparison, the tissues examined were grouped into four contiguous areas, shown within the enclosed blocks above (upper airway, Balt, lung parenchyma and alveolar spaces), each of which could be conveniently scanned in a single low power microscope field. Within each area, staining frequencies are scored from ++++ (highest average counts per field) to ± (occasional positive cells only). For comparisons of staining densities between the four areas, refer to photomicrographs below.

* Based upon observations of isolated alveolar cells prepared by lavage (refer to Table 1 for precise figures).

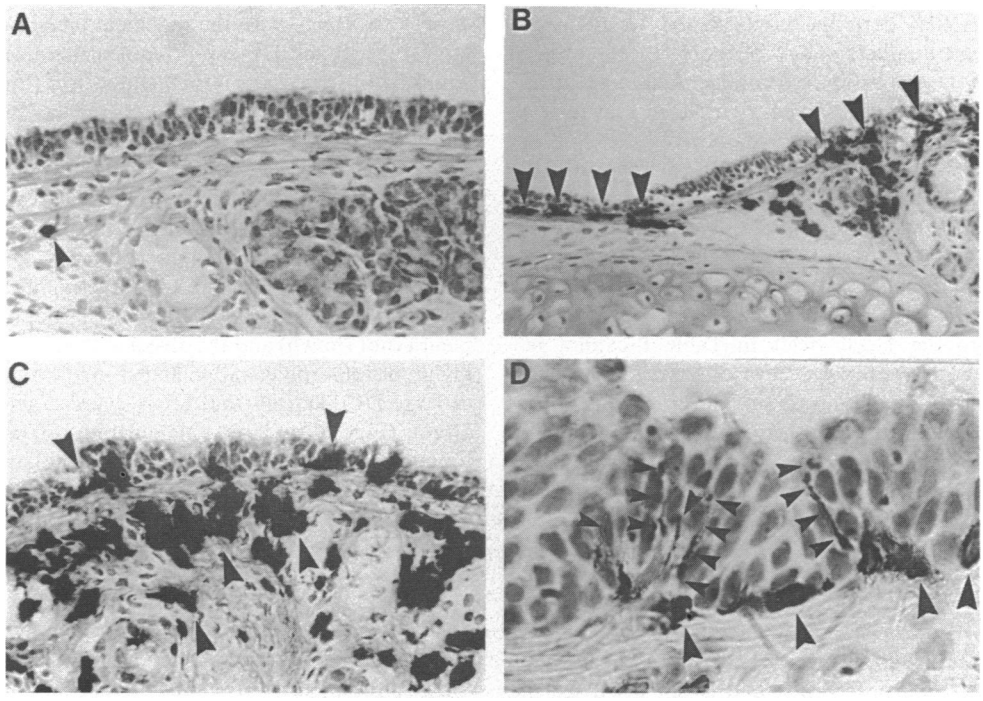

Figure 1. Distribution of Ia^+ cells in tracheal tissue. A, control; note occasional cell staining for endogenous peroxidase (× 101). B, Ia^+ cells within tracheal epithelium; note close association with epithelial basement membrane (× 58·5). C, Ia^+ cells (e.g. arrows) within epithelium, and in underlying mucosa, in particular adjacent to serous glands (Fig. 2B) (× 101). D, High power view of Ia^+ cells closely associated with epithelial basement membrane (large arrows); note Ia^+ dendritic processes (small arrows) between epithelial cells (× 255).

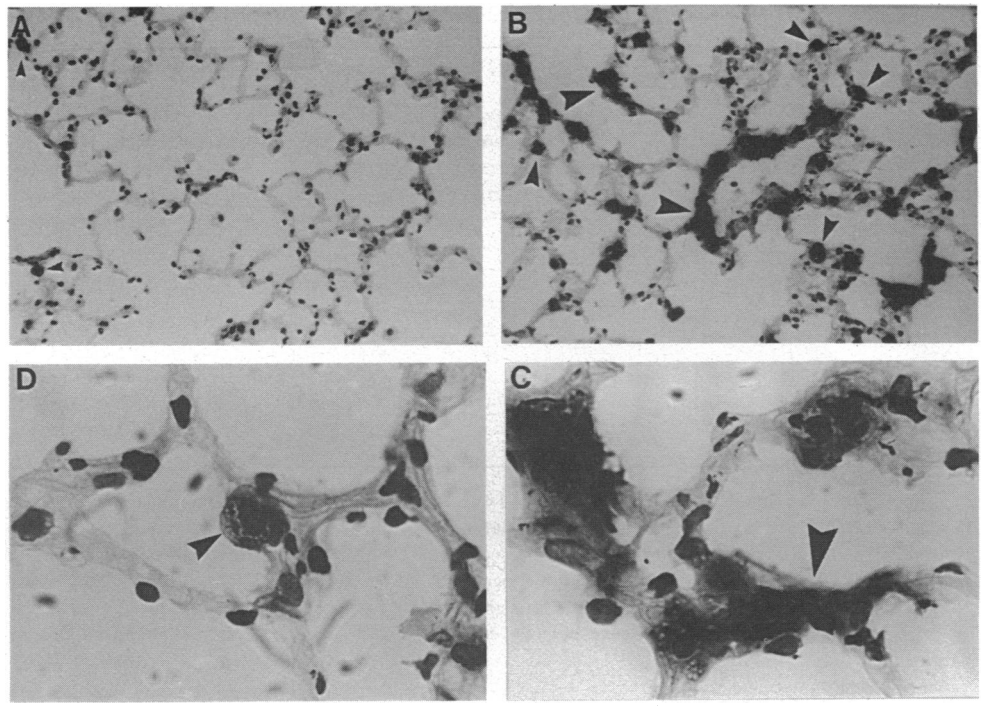

Figure 2. Ia$^+$ cells in peripheral lung tissue. A, control; note occasional endogenous peroxidase positive cells (× 58·5). B, large and small Ia$^+$ cells in alveolar septal walls (× 58·5). C, Ia$^+$ DC-like cell spanning two adjacent alveoli (× 255). D, vacuolated Ia$^+$ cell attached to air-side of septal wall (× 255).

It can be seen that both the tracheal and lung digests contained significant numbers of Ia$^+$ cells and T cells, which were not found in lavage samples. Prominent staining of all three cell preparations was observed with MoAbs against a number of markers reportedly found on macrophages and/or DC. Figures also are included in Table 1 for the adherent (i.e. macrophage-enriched) component of the lavage and lung digest populations. Under the conditions employed (see legend) ≥97% of alveolar macrophages were glass adherent, as opposed to 60–70% of lung digest macrophages.

The contribution of bronchus-associated lymphoid tissue (BALT) T cells to the T-cell yields in Table 1 cannot be calculated precisely. However, we have observed that T-cell yields per gram wet weight of lung are similar for samples taken from the periphery of the organ (where little BALT is found), and for samples taken in the vicinity of the larger airways (data not shown), and we thus conclude that the BALT population is a relatively minor component of the overall lung T-cell pool.

Tissue distribution of individual cell populations in rat lung

Table 2 comprises a synopsis of our findings, employing the major T-cell, DC and macrophage markers. BALT is included here for comparative purposes only, and is not discussed in detail in the text below.

Class II MHC antigen-bearing cells

As illustrated in Fig. 1, tracheal tissue contained large numbers of Ia$^+$ cells. Two separate populations were discernible. The first comprised DC-like cells spaced at relatively regular intervals along the epithelium (Fig. 1B). These cells often appeared to be closely associated with the basement membrane of the epithelium (Fig. 1B and D), and prominently stained Ia$^+$ 'dendrites' could be observed at high magnification (Fig. 1D) and often reached as far as the airside of the epithelium. A second population of highly pleiomorphic Ia$^+$ cells was observed in the underlying mucosa, and in particular appeared concentrated in the vicinity of serous glands (Figs 1B and C); the epithelium overlying these areas usually contained larger numbers of Ia$^+$ cells than was found within intervening sections.

Ia$^+$ cells were also found in large numbers in BALT, and throughout the lung parenchyma. The population within the pleura (not shown) and the alveolar septal walls (Fig. 2B) were highly pleiomorphic, and included small rounded cells, as well as large DC-like cells that often spanned across two adjacent alveoli (Fig. 2C). Large conglomerates of Ia$^+$ cells were also found adjacent to bronchioles and small blood vessels. No Ia$^+$ cells were found free in the alveolar spaces, or in lavage samples; however, significant numbers of vacuolated, Ia$^+$ cells were found attached to the air side of alveolar walls (Fig. 2D).

Macrophage and DC markers

The MoAb ED1 stained distinct cell types in different areas of the respiratory tract. This marker appeared on large DC-like cells in BALT (not shown) as reported previously (Dijkstra *et al.*, 1985), and similar ED1$^+$ cells were observed along the basement membrane of the airway epithelium (Fig. 3A). In the lower lung, large free cells (putative alveolar macrophages) in the alveolar spaces stained strongly (Fig. 4A), and similar cells (often vacuolated) were observed attached to the alveolar septal walls (Fig. 2D). ED1$^+$ cells also appeared within the septal walls (Fig. 4A), and these were small and readily distinguishable from

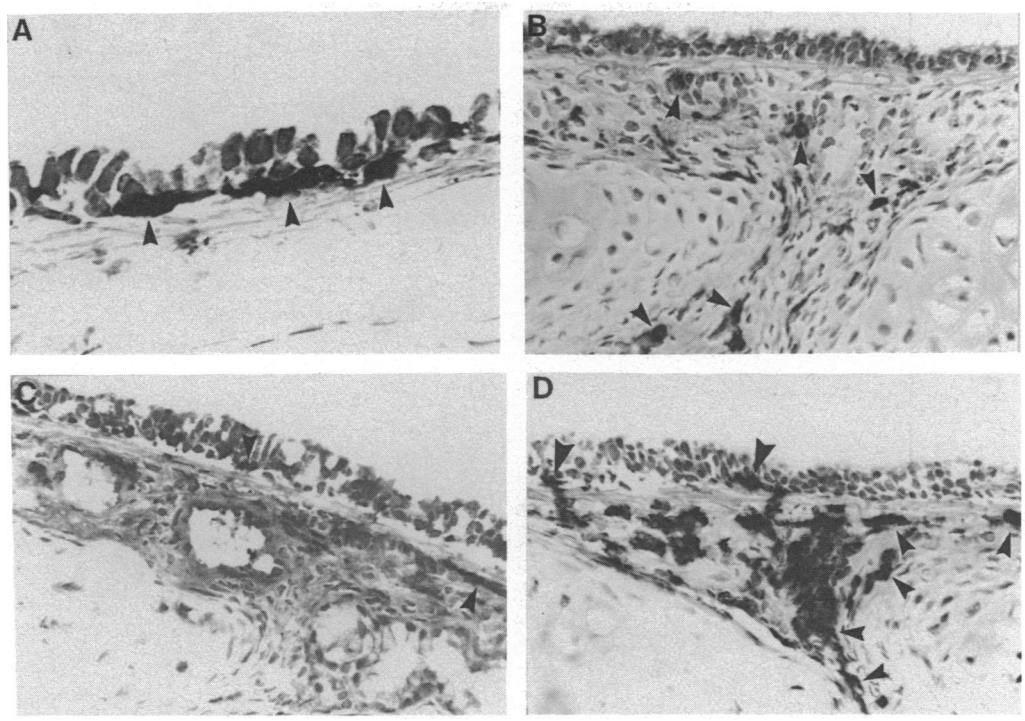

Figure 3. Expression of macrophage and DC markers on tracheal cells. A, DC-like ED1$^+$ cells associated with epithelial basement membrane (× 255). B, ED2$^+$ cells (arrowed) were seen occasionally below, but not within, the epithelium (× 101). C, OX-41$^+$ cells were extremely rare (two only visible here; relatively high background staining prevents clear reproduction in B&W) (× 101). D, OX-42$^+$ cells within (large arrows) and below (e.g. small arrows) the epithelium (× 101).

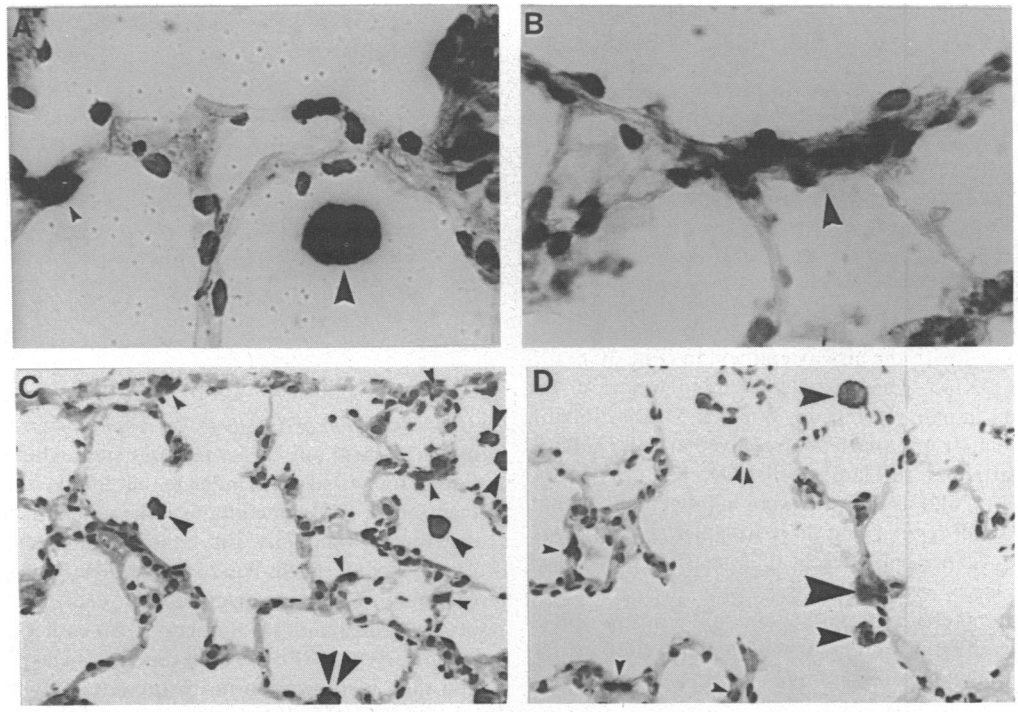

Figure 4. Expression of macrophage and DC markers on parenchymal lung cells. A, alveolar macrophage (large arrow) and septal wall cell (small arrow) stained with ED1 (× 255). B, DC-like ED2$^+$ cell within alveolar septal wall (× 255). C, OX-41$^+$ within the pleura and septal walls (small arrows), free within the alveolar spaces (large arrow) and occasionally attached to septal walls (two arrows) (× 101). D, OX-42$^+$ cells within the septal walls (small arrow), and both free in the alveolar spaces (medium arrow) or attached to the wall (large arrow); note OX-42$^-$ free alveolar macrophage (two arrows) (× 101).

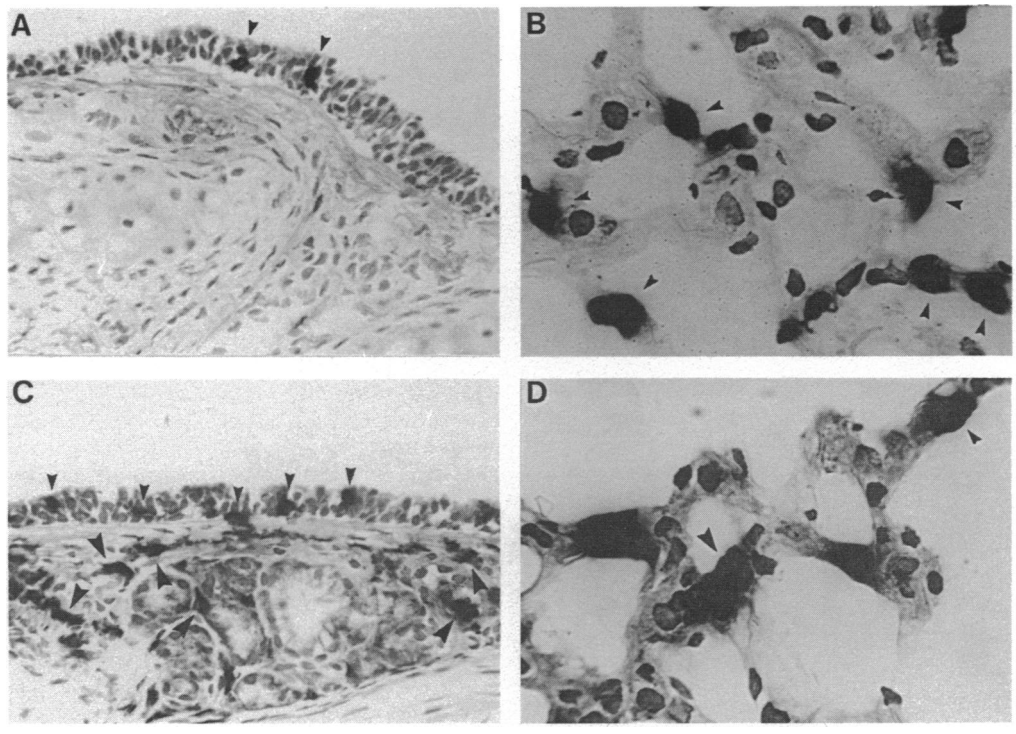

Figure 5. Expression of T-cell markers on tracheal and parenchymal cells. A, T cells stained with OX-19/52 in tracheal epithelium (×101). B, OX-19/52$^+$ cells in alveolar septal walls (×101). C, W3/25$^+$ cells within (small arrows) and below (e.g. large arrows) the tracheal epithelium (×101). D, both small, round (small arrows) and larger, irregular (large arrows) W3/25$^+$ cells in alveolar septal walls.

those described above. DC-like ED1$^+$ were observed in peribronchial and perivascular areas. ED2$^+$ cells were not observed within the tracheal epithelium (Fig. 3B) or in the free alveolar population. These cells were prominent in peribronchial and perivascular areas, and were also found in significant numbers in the pleura and within the septal walls (Fig. 4B), the latter exhibiting a characteristic DC-like morphology.

The MoAb OX-41 stained all the free alveolar macrophage population (Fig. 4C), comparable to that seen with ED1. OX-41$^+$ cells were also occasionally noted adherent to the air side of the alveolar septa (less frequently than then adherent cells stained by OX-6 and ED1), and a few small OX-41$^+$ cells were observed in the pleura and within alveolar walls. Numerous OX-41$^+$ cells were seen in BALT, but only occasional cells were observed associated with the airway epithelium (Fig. 3C).

The MoAb OX-42, which is equivalent to Mac-1 in the mouse or OKM1 in man (Robinson, White & Mason, 1986b), stained approximately one quarter of free alveolar cells (Table 2; Fig. 4D), in particular the larger cells. OX-42$^+$ cells in the alveolar walls (Fig. 4D) and pleura were highly pleiomorphic, including both small regular and large irregular shaped types. These cells were also present in tracheal tissues (Fig. 3D), where they exhibited large, irregular morphology; they were also found in small numbers in the epithelium, and a proportion of the latter displayed typical DC morphology, and close association with the epithelial basement membrane.

T-cell markers

The pan T cell MoAbs OX-19 and OX-52 stained small numbers of cells in tracheal tissue (Fig. 5A), and much larger numbers of small, regular shaped cells stained within the septal walls (Fig. 5B) and in BALT (not shown). OX-8$^+$ cells (not shown) exhibited a similar distribution throughout BALT and alveolar septa.

The MoAb W3/25, originally identified as staining T-helper cells (Mason *et al.*, 1983), reacted with numerous cells in tracheal and alveolar septal tissue (Fig. 5C and D). The majority of the W3/25$^+$ septal cells were large and irregular in shape, and may include some macrophages and DC, which are now known to express an identical marker (Barclay, 1981; Robinson *et al.*, 1986b). Small W3/25$^+$ cells in the tracheal epithelium were similarly pleiomorphic and included DC-like cells associated with the basement membrane; the majority of the underlying mucosal population were of the large, irregular type.

DISCUSSION

Distribution of T cells in the respiratory tract. These experiments confirm our earlier findings employing enzymatic digestion of lung tissue, and indicate that the extravascular compartment of the rat lung contains large numbers of T cells that are distributed randomly throughout the alveolar septal walls. These include both W3/25$^+$ (putative T-helper) and OX-8$^+$ (putative T-suppressor/cytotoxic) cells. T cells were not observed in significant numbers in an earlier immunohistochemical survey of SPF rat lung (Simecka, Davis & Cassel, 1986), and their identification in the present study may be related to our use of a biotin/streptavidin amplification system, which maximizes immunoperoxidase staining. Additionally, the use of a mixture of MoAb against rat T-cell markers (OX-19/52) improved staining significantly over that obtained with either antibody alone.

Table 3. Distribution of cells with DC-like morphology, revealed by immunoperoxidase staining with a panel of MoAb

MoAb	Relative frequency within: Tracheal epithelium	Tracheal mucosa	Alveolar septal wells
OX-6	++++	++++	++++
ED1	+++	++	−
ED2	−	−	++
OX-41	±	+	±
OX-42	+	+++	++
W3/25	+++	+++	++

Data shown reflect relative frequency of positively staining cells within low power microscope fields, as in Table 2.

In the upper respiratory tract, isolated T cells were observed within the airway epithelium and mucosa. The major T-cell population in this area was clearly that within the BALT; however, results from other studies suggest that large number of T cells may accumulate in the airway epithelium and mucosa during inflammation (Erlander, Ahlstedt & Nygren, 1984).

Distribution of macrophages and DC-like cells. The interpretation of results obtained with the panel of MoAb against DC and macrophage markers is limited by the fact that many of these appear to react with both cell types. Consideration of the physical properties of the stained cells (Table 3) assists significantly in clarifying this situation. Thus, the cells in tracheal sections which display classical DC-like morphology express the surface phenotype OX-6$^+$, ED1$^+$ and W3/25$^+$, analogous to their counterparts in lymph (Pugh, MacPherson & Steer, 1983; Dijkstra *et al.*, 1985; Mayrhofer *et al.*, 1986; Robinson *et al.*, 1986b). These cells stain relatively weakly for the OX-41 and OX-42 markers, which are present on $>50\%$ of lymph DC (Robinson *et al.*, 1986b), and this may reflect a subtle phenotypic difference resulting from their maturation within the microenvironment of the airway epithelium.

A more distinct difference was noted between these DC-like cells and their counterparts in the alveolar walls. Unlike the airway epithelial DC, those from the alveolar septa stained strongly for the ED2- surface marker. Apart from Kupffer cells in the liver, the only 'branched' cell previously reported to react with the ED2 MoAb appears to be a prominent dendritic population in the thymic cortex (Dijkstra *et al.*, 1985), which may be involved in regulation of thymocyte maturation (Beller & Unanue, 1978).

It should also be noted that obvious heterogeneity exists within, as well as between, these two respiratory tract DC populations. As shown in Table 3, if positive staining for Ia (i.e. OX-6$^+$) is taken as the bench mark, then the surface molecules defined by the MoAbs ED1, ED2, OX-42 and W3/25, appear on only 25–75% OX-6$^+$ DC-like cells. A similar order of heterogeneity has been noted in DC populations from rat lymph (Pugh *et al.*, 1983; Mayrhofer *et al.*, 1986), and indeed amongst macrophages (Sorg, 1982; Bursuker & Goldman, 1983; Dijkstra *et al.*, 1985).

In relation to macrophages (Table 3; column 1, Table 1), expression of Ia appears minor in comparison to the markers defined by ED1 and OX-41/2. This is particularly the case amongst the alveolar macrophages, where Ia expression appears restricted solely to a subset of cells which are tightly adherent to the air-side of the alveolar surface (Fig. 2D), and which resist endobronchial lavage. This implies that Ia induction in alveolar macrophages accompanies the attachment process, and may thus represent an 'activation' marker. The situation with respect to tracheal macrophages is less clear, as the frequency of Ia$^+$ cells exceeded those staining for markers such as OX-41 and OX-42 (Table 1, Figs 1 and 3); double-staining procedures will be required to clarify this issue.

Implications for studies on immune regulation in the lung. A series of recent reports have identified Ia$^+$ DC as major APCs in the cell preparations derived from tissues of the upper (Sertl *et al.*, 1986; Holt *et al.*, 1987) and lower respiratory tract (Holt *et al.*, 1985a) of experimental animals, and morphologically similar cells have been recognized in human transbronchial biopsies (Campbell, Poulter & Dubois, 1985). The present study has demonstrated the presence of cells with relevant morphological and surface phenotypical properties, at strategic sites in lung tissue for engagement with incoming antigen, notably within the airway epithelium and within the alveolar septal walls. The former may be the respiratory tract equivalent of the DC population we have recently shown to be involved in antigen transport from the gut wall, and subsequent presentation to T cells in the draining lymph nodes (Mayrhofer *et al.*, 1986), and if so they may play a crucial role in determining the nature of the immune response to inhaled antigens.

The DC-like cells detected in sections of respiratory tract tissues appear to uniformly express high levels of Ia, but are heterogeneous with respect to the other surface markers employed here. Of the latter, the ED1/2 markers appear to be most useful, as they distinguish between alveolar and airway epithelial DC, and thus provide a potential tool for isolation of the two populations for functional studies.

It is also evident that these DC-like cells are intimately associated with a heterogenous population of pleiomorphic cells which express one or more macrophage surface markers, and consequently the interactions which have been shown to occur between these two cell types during antigen-driven T-cell activation *in vitro* (Holt *et al.*, 1985a; Holt *et al.*, 1987), may potentially occur under comparable circumstances *in vivo*. Thus the further investigation of these two cell types, in particular questions relating to their turnover *in situ* and possible functional heterogeneity, appears warranted.

This study has also drawn attention to the presence of a large T-cell population within the alveolar septal walls. In SPF rats, this population is at least five times larger than the peripheral blood T-cell pool (Holt *et al.*, 1985b), and in man is at least equivalent to the latter (Holt *et al.*, 1986). We currently know little about these cells, but may speculate from their occurrence as single, isolated cells, that they are immigrants from the blood as opposed to a local self-sustaining population. Unresolved issues include their precise location within the lung wall (marginating within vessels versus extravasated?), their origin and eventual fate, and the nature of the selection process (if any) which caused them to be initially trapped in the tissue vascular bed (e.g. see Hall, 1985). We are presently engaged in studies on the half-life of these T cells, and are performing limiting dilution

analyses of their function(s), as a preliminary approach to these important questions.

ACKNOWLEDGMENTS

This work was supported by the National Health and Medical Research Council of Australia and Princess Margaret Children's Medical Research Foundation. This is Publication No. 266 from the PMCMRF Clinical Immunology Research Unit.

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