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Constitutive expression of MHC and adhesion molecules by alveolar epithelial cells (type II pneumocytes) isolated from human lung and comparison with immunocytochemical findings

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SUMMARY

Highly purified populations of alveolar epithelial cells (type II pneumocytes) were isolated from human lung specimens. These cells were characterised histochemically, by demonstrating the presence of intracellular alkaline phosphatase, and morphologically, by electron microscopic demonstration of lamellar bodies and microvilli. Expression of the epithelial glycoprotein HEA-125, of MHC class I and class II (HLA-DR, -DP and -DQ) antigens and of the intercellular adhesion molecules ICAM-1, VCAM-1, LFA-3 and B7 was quantified by flow cytometry. Comparison was made between the expression of these molecules by isolated type II cells and by alveolar epithelium in normal human lung tissue after immunocytochemical staining of frozen sections of donor lung.

Isolated type II pneumocytes expressed HEA-125 and class I MHC molecules and the class II MHC molecules HLA-DR and -DP; HLA-DQ was not detected. The intercellular adhesion molecule ICAM-1 was expressed constitutively at low levels but there was minimal expression of VCAM-1, LFA-3 and B7.

It was not possible to differentiate type II cells from the predominant type I pneumocytes on frozen sections. Alveolar epithelium expressed HEA-125, class I MHC antigens, the class II molecules HLA-DR, and -DP and the intercellular adhesion molecule LFA-3. Expression of the adhesion molecules ICAM-1, VCAM-1 and B7 was variable. As with the isolates, HLA-DQ was not observed on alveolar epithelium.

In conclusion, a reproducible method for the isolation of pure populations of human type II pneumocytes has been developed. These cells were not damaged by the isolation procedure. It is not known whether alveolar epithelium can present antigens to T lymphocytes. However, the expression of potentially immunogenic class II MHC antigens and of intercellular adhesion molecules may facilitate pulmonary inflammation.

Key words: type II pneumocytes, class II MHC, epithelium, lung, immunogenicity

INTRODUCTION

Human airway epithelial cells are vital for the maintenance of the pulmonary air-blood barrier. Gaseous diffusion occurs across alveolar type I pneumocytes while pulmonary surfactant, essential for reducing the surface tension of alveolar surfaces, is secreted by type II pneumocytes. Type II pneumocytes are the progenitors of type I cells.

In addition to their important physiological functions, there is a body of evidence to indicate that type II pneumocytes have a role to play in the regulation of immune responses in the lung. Type II pneumocytes are known to express class II major histocompatibility complex (MHC) molecules in vivo; these molecules constitute the ligand for the antigen receptor on helper T lymphocytes (Glanville et al., 1989; Peters et al., 1990). Expression of class II molecules is usually limited to specialised cells of the immune system whose role is to present foreign antigen to T cells. However, expression of class II MHC molecules can be induced on parenchymal cells such as epithelial cells, endothelial cells and fibroblasts, following the appropriate stimulation (Fabre, 1991).

Chronic bronchitis (inflammation of the large airways) is associated with increased expression of class II MHC antigens by the epithelial cells of human airways (Popp et al., 1992). The expression of class II antigens by type II pneumocytes is raised in animal models of lung inflammation caused by allograft rejection (Chang et al., 1990; Romaniuk et al., 1987) and by treatment with paraquat (Nakayama et al., 1992), bleomycin (Struhar et al., 1990) and quartz crystals (Struhar et al., 1989). Intraperitoneal administration of gamma interferon (IFN-γ) upregulates class II MHC molecule expression on type II pneumocytes in rats (Schneeberger et al., 1986).

Freshly isolated rat type II pneumocytes express class II MHC antigens, which are not detectable during subsequent culture. However, class II molecules can be induced in culture

by treatment of the type II cells with IFN-γ (Harbeck et al., 1988). The expression of class II molecules by alveolar epithelium does not appear to be a response to subclinical inflammation elicited by environmental antigens, since type II pneumocytes of pathogen free rats constitutively express these molecules (Steiniger and Sickel, 1992).

Relatively little is known about the expression of adhesion molecules by respiratory epithelia. The LFA-1 (CD11a/CD18) → ICAM-1 (CD54), CD2 → LFA-3 (CD58), VLA-4 (CD29/CD49d) → VCAM-1 and CD28 → B7 interactions stabilise the binding of lymphocytes to MHC antigen-expressing cells. Adhesion molecule binding also causes T cell signal transduction, which may modulate cell activation. Blockade of adhesion molecule function prevents T cell proliferation and the generation of cytotoxic lymphocytes in mixed leukocyte cultures (Hildreth and August, 1985; Harding et al., 1992; Suranyi et al., 1991).

Human bronchial epithelium expresses ICAM-1, which can be upregulated by the inflammatory cytokines IL-1 and TNF-α (Tosi et al., 1992). The expression of ICAM-1 is known to correlate with increased binding of activated polymorphonuclear cells (PMN) to monolayers of bronchial epithelial cells. This binding can be inhibited by monoclonal antibodies specific to ICAM-1 or its ligand on PMN. The expression of ICAM-1 has not been detected on freshly isolated rat type II pneumocytes, but expression has been observed in vitro following their differentiation into type I pneumocytes (Christensen et al., 1993a,b; Burns et al., 1991). Many inflammatory and parenchymal lung diseases involve damage to the epithelium; increased expression of MHC and adhesion molecules may be contributory factors to the development of this pathology.

The majority of studies of alveolar epithelium have been performed with rodents. However, species differences between lung tissue of man and rodent do exist (Plopper et al., 1980a,b; Hyde et al., 1991) and the clinical relevance of extrapolating data from animals may be questionable. The aim of this study was to advance the understanding of the immunobiology of human alveolar epithelium. Previous methods of isolating pure populations of type II pneumocytes from normal (asymptomatic) human lung were improved. The expression of the class II MHC molecules HLA-DR, HLA-DP and HLA-DQ, and the adhesion molecules ICAM-1, LFA-3, VCAM-1 and B7 by the isolated cells was then quantified by flow cytometry. The influence of the isolation procedure on expression of these molecules was determined by comparison with tissue sections that had been stained immunocytochemically to identify the same antigens.

MATERIALS AND METHODS

Isolation of type II pneumocytes

Six samples of normal human lung were used in this study; three specimens were obtained from healthy donor lungs that were not used for organ transplantation (1 female, 2 males; mean age 26 years; 2 smokers; designated TX1, TX2 and TX3), and three specimens that were recovered during lobectomy at sites distal to any tumour (3 males; mean age 68 years; 2 smokers; designated L1, L2 and L3). It was possible to store tissue samples overnight at 4˚C following airway perfusion with Euro-Collins solution (Pharmacy Manufacturing, Newcastle General Hospital, Newcastle upon Tyne, UK).

Type II pneumocytes were isolated using a modification of the method of Bingle et al. (1990). Briefly, the specimen was perfused via convenient airways with 0.15 M Saline (British Drug Houses, Poole, Dorset, UK). The lung was then perfused with 20-40 ml of 0.5% (w/v) bovine pancreatic trypsin (T-8003, Sigma Chemical Company, Poole, Dorset, UK) in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS; Northumbria Biologicals, Cramlington, Northumberland, UK) or, in the case of TX3, with 30 units/ml porcine pancreatic elastase (E1250, Sigma). The specimen was incubated at 37˚C for approximately 30 minutes and was then chopped finely in the presence of foetal calf serum (FCS; 5 ml, Gibco, Paisley, Scotland) and DNase I (15 ml, 500 μg/ml, Boehringer Mannheim, Lewes, East Sussex, UK). The tissue was transferred to a flask with an additional 20 ml of HBSS and was shaken for 5 minutes at 37˚C. A cell-rich fraction was obtained by sequential filtration through gauze, 125 μm and 30 μm nylon meshes (Lockertex, PO Box 161, Warrington, Cheshire, UK); this was then centrifuged (400 ***g***, 10 minutes). The resultant pellet was resuspended in DMEM (Gibco) containing 10% FCS (v/v; 2 ml volume) and was layered onto a continuous Nycodenz density gradient (Nycomed Pharma AS, Oslo, Norway). These gradients were prepared by diluting a Nycodenz solution (1.15 g/ml) with Tris-HCl buffer at pH 7.5 in ratios of 1:0, 3:1, 1:1, 1:5 to produce densities of 1.15, 1.11, 1.07, 1.025 g/ml, respectively. Samples (2 ml) of each density were layered by decreasing density, the tube was placed horizontally and the solutions were allowed to diffuse for 1 hour at room temperature (RT). The density gradient was centrifuged for 20 minutes at 1500 ***g*** and the upper band containing a mixture of type II pneumocytes and alveolar macrophages was recovered, washed twice by centrifugation in HBSS (Viscardi et al., 1992) and resuspended in DMEM (containing 10% (v/v) FCS; 10-15 ml). Alveolar macrophages were depleted by incubation with carbonyl iron (0.2 g; ISP (GB), Wythenshawe, Manchester, UK) for 1 hour at 37˚C with regular mixing. The carbonyl iron was removed with a magnet (Dynal MCP-1, Wirral, Merseyside, UK) and the process was repeated. Type II pneumocytes were cryopreserved in FCS containing 10% dimethylsulphoxide (DMSO; v/v, Sigma) and stored in liquid nitrogen prior to use.

Characterisation of type II pneumocytes

Alkaline phosphatase

Cytocentrifuge (Cytospin 3; Shandon Scientific Ltd, Runcorn, Cheshire, UK) cell smears were allowed to air dry and were stained for 15 min at RT. The stain was prepared by dissolving 10 mg naphthol AS bi-phosphate (Sigma) in 40 μl DMSO and was diluted in 10 ml of 0.125 M 2-amino-2-methyl propanol buffer (Sigma; pH 8.9) containing 10 mg fast red (Sigma). The slide was washed and counterstained in 1% methylene green (Sigma) for 30 seconds and was mounted in aqueous medium.

Transmission electron microscopy

Cells were fixed in 2% glutaraldehyde (Agar Scientific Ltd, Essex, UK), washed three times in phosphate buffered saline and serially dehydrated in acetone (British Drug Houses, Dorset, UK). The tissue was post-fixed in 1% osmium tetroxide (Agar Scientific Ltd.) and embedded in an Araldite resin (medium premix kit, TAAB Laboratories Equipment Ltd., Berks, UK). Semi-thin sections (1 μm) were cut using an ultramicrotome (Ultracut E, Reichert-Jung) with a diamond knife and were stained with toluidine blue for light microscopic examination. Ultrathin sections (80 nm) were mounted on copper grids, were electron contrasted with uranyl acetate (1.5 hours, 30˚C, Reichert-Jung) and lead citrate (40 minutes, 20˚C, Reichert-Jung; LKB-Wallac, 2168 Ultrastainer), and were examined with a transmission electron microscope (JEM-100S, JEOL(UK) LTD, Herts, UK).

Surface antigen expression of alveolar epithelium

Flow cytometry

The expression of cell surface antigens was measured by staining

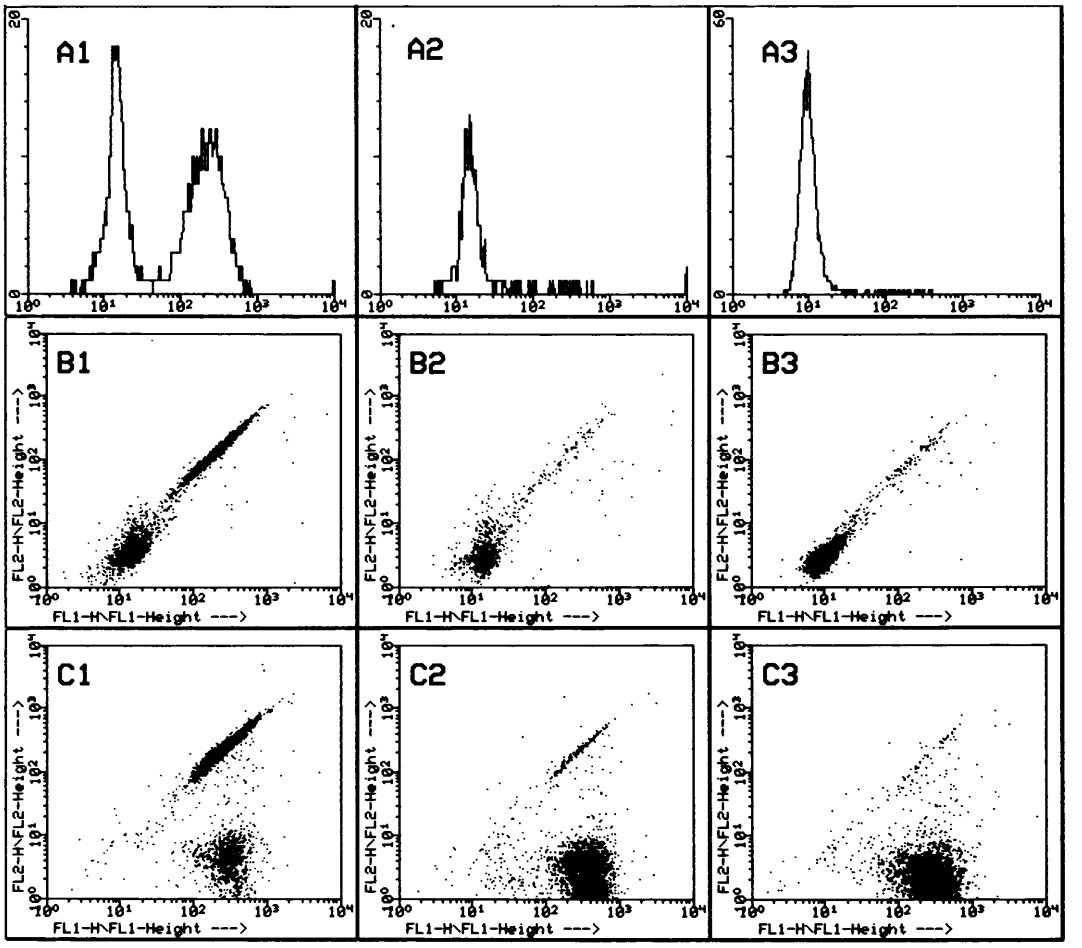

Fig. 1. Effect of carbonyl iron on the removal of autofluorescent/CD45-positive macrophages from the type II pneumocyte preparation. (A) Fluorescence histograms of unstained cell preparations (FL1, FITC channel). (B) Dual parameter dot plots showing autofluorescence of unstained cell preparations (FL1 and FL2, PE channel). (C) Dual parameter plots of cell populations stained with anti-HEA-125 + GAMFITC-conjugated antibodies (FL1) and anti-CD45 PE-conjugated antibodies (FL2). (1) Mixed cell populations before carbonyl iron treatment. (2) Cell preparation following 1 treatment with carbonyl iron. (3) Cell preparation following 2 treatments with carbonyl iron.

purified type II cells with optimal dilutions of a panel of murine monoclonal antibodies (4˚C, 30 minutes) followed by a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (Sigma F-0257, 4˚C, 30 minutes). Each cell preparation was co-stained with an antibody specific for leukocyte common antigen (CD45 conjugated with phycoerythrin, MCA 87PE, clone F10-89-4 IgG2a; Serotec, Oxford, UK). The antibodies used were specific for HEA-125 (Serotec), HLA -A, -B, -C (clone W6/32; ECACC, Salisbury, UK), HLA-DR (clone L234; Becton Dickinson, Oxfordshire, UK), HLA-DP (clone B7/21; Becton Dickinson) and HLA-DQ (clone SK10; Becton Dickinson), B7 (kindly provided by Dr A Freedman, Dana-Farber Cancer Institute, Boston, MA, USA), ICAM-1 (CD54, BBA3, clone BB1G-I1 IgG1; British Biotechnology Ltd, Abingdon, Oxon, UK), VCAM-1 (BBA5; British Biotechnology Ltd), and LFA-3 (CD58, BRIC 5; Blood Products Laboratory, Hertfordshire, UK). A non-specific antibody was used as a negative control in each labelling experiment (IgG1, X931; Dako Ltd, High Wycombe, Bucks, UK). The cells were examined by flow microfluorimetry (FACScan; Becton Dickinson), and the FITC-stained cells were detected by measuring green light emitted at 530 nm (FL1 channel). The antigen expression of any contaminating alveolar macrophages was effectively ignored by the removal of highly autofluorescent and CD45-positive cells detected by the FL2 channel (red light emitted at 575 nm). The amount of FITC label, and therefore specific antibody, bound by the remaining CD45 negative cells was then quantified.

Immunohistochemistry

Fragments of lung (TX1, TX2 and TX3) were snap-frozen in liquid nitrogen-cooled isopentane. Tissue sections, 5 μm in thickness, were air dried, fixed in acetone and reacted with the panel of monoclonal antibodies used above and with a mixture of antibodies specific for cytokeratin (clone 5D3; Novocastra Laboratories, Newcastle upon Tyne, UK; and clone LP34; Dako Ltd, High Wycombe, Bucks, UK). A standard indirect immunoperoxidase technique was employed (Green et al., 1989). Peroxidase development was performed with nickel-modified diaminobenzidine, and post-development enhancement was performed with Tris/cobalt. The presence or absence of HEA-125, cytokeratin, class I MHC antigens (HLA-A,B,C), HLA-DR, HLA-DP, HLA-DQ, ICAM-1, VCAM-1, LFA-3 and B7 was noted on the alveolar epithelium.

RESULTS

Two distinct populations of cells were initially recovered from

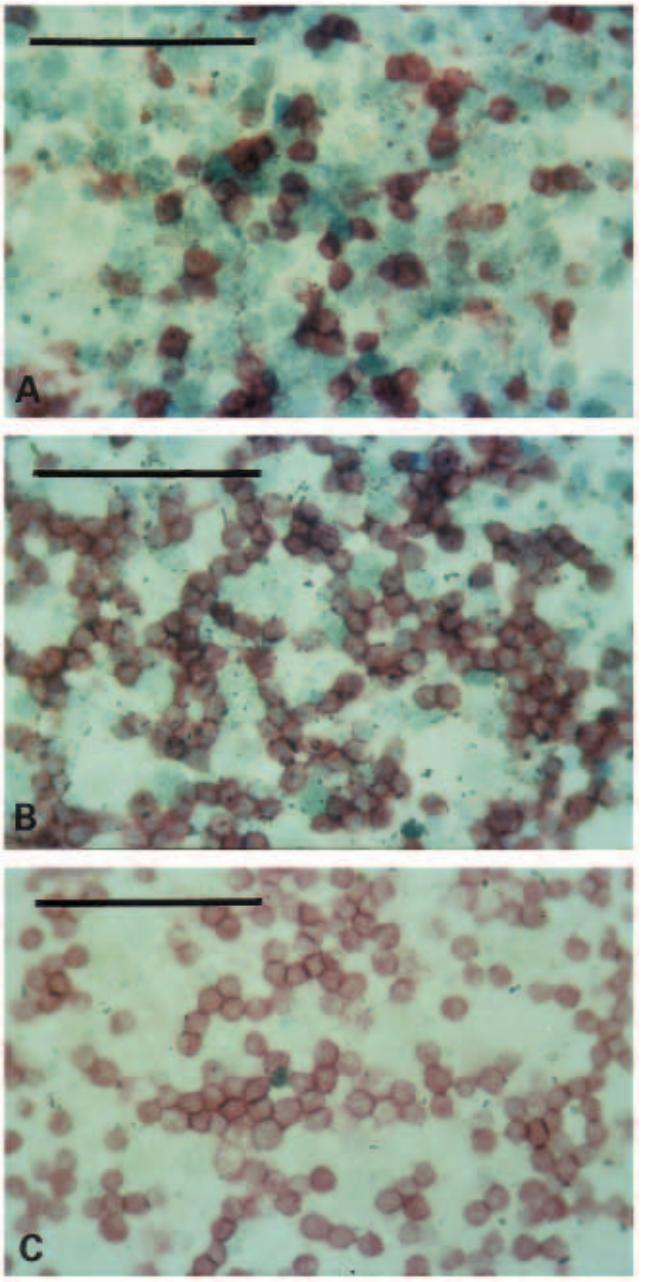

Fig. 2. Cytospin preparations showing alkaline phosphatase-positive cells from human lung: (A) without carbonyl iron; (B) after the first carbonyl iron; (C) after the second carbonyl iron. Bars, 50 μm.

the density gradient as demonstrated by flow cytometry (Fig. 1, A1) and alkaline phosphatase activity (Fig. 2). One population was less autofluorescent, expressed the epithelial cell surface glycoprotein HEA-125, but not the macrophage cell surface marker CD45 (Fig. 1, C1). These cells were characterised by the presence of intracellular alkaline phosphatase (Fig. 2) and by lamellar bodies and microvilli at the electron microscope level (Fig. 3A) demonstrating that they were type II pneumocytes. Cells in the other population were extremely autofluorescent, did not stain positively for alkaline phosphatase, and were subsequently found to be alveolar macrophages following cell sorting (FACS 420, Becton Dickinson) and transmission

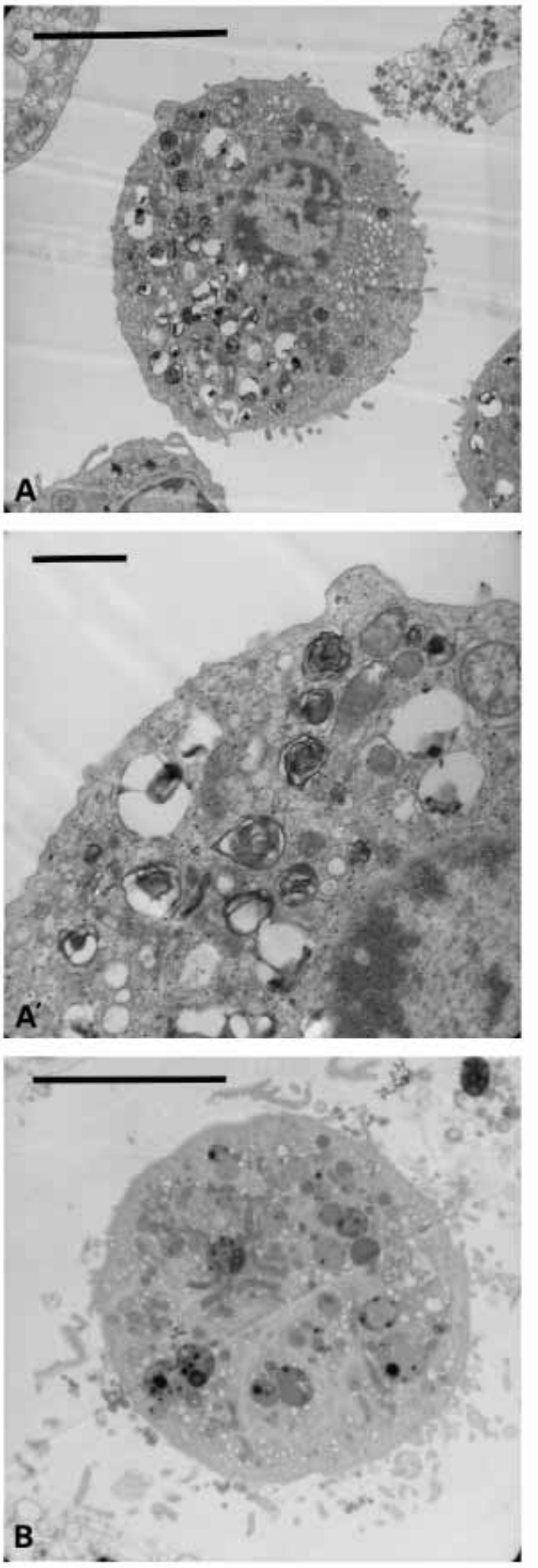

Fig. 3. (A) Transmission electron micrographs of type II cells showing lamellar bodies at lower (A) and higher (A′) power. Bars: 5 μm and 1 μm, respectively. (B) Transmission electron micrograph of an alveolar macrophage. Bar, 5 μm.

Table 1. The effect of serial carbonyl iron treatment on the proportion of autofluorescent and CD45 +ve cells in the population of isolated pulmonary cells

	Without carbonyl iron		X1 Carbonyl iron		X2 Carbonyl iron	
Cell characteristics	Median fluorescence	% +ve	Median fluorescence	% +ve	Median fluorescence	% +ve
Auto fluorescent	220	56	254	15	172	4
Non fluorescent	15	44	15	85	10	96
CD45 +ve	254	69	289	6	161	2
CD45 –ve	4	31	2	94	1	98

electron microscopy (Fig. 3B). Carbonyl iron was very efficient for the removal of alveolar macrophages from the type II pneumocytes in the preparation (Figs 1 and 2). In the example shown 69% of the cells were labelled with PE-conjugated anti-CD45 and were detected in the FL2 channel by flow cytometry (Table 1; Fig. 1, C1). After one treatment with carbonyl iron the proportion of these cells dropped to 6% of the population (Table 1; Fig. 1, C2); a second treatment with carbonyl iron reduced the proportion of CD45-positive cells to 2% of the population (Table 1; Fig. 1, C3). The CD45 negative cell population uniformly expressed the epithelial glycoprotein HEA-125 (Fig. 1, C3). At this stage any contaminating alveolar macrophages were electronically excluded during antigen quantification assays.

The use of different enzymes during the preparation had a an effect on the final cell yield. When the lung tissue was incubated with elastase, type II pneumocytes constituted only 20% of the total cell population before carbonyl iron treatment (n=7, data not shown); incubation with trypsin increased the yield of type II pneumocytes to about 50% of the total cell population. The results presented in this report were obtained using type II pneumocytes isolated using trypsin, with the exception of cells from specimen TX3, which were prepared after elastase digestion. Recent studies in our laboratory have demonstrated that an approximate fivefold increase in cell yield is achieved by the use of 1.04-1.09 g/ml discontinuous Percoll gradients rather than Nycodenz during the density gradient centrifugation step (Bingle et al., 1990).

Isolated human type II pneumocytes expressed the epithelial glycoprotein HEA-125, ICAM-1, class I MHC molecules (HLA -A,B,C) and the class II MHC molecules HLA-DR and -DP. The class II antigen HLA-DQ was not detected on the isolated type II cells. There was limited expression of B7, VCAM-1 and LFA-3 (Table 2). There was very little auto fluorescence by the isolated type II cells, or background staining with the control antibody (Fig. 4). By comparison, representative fluorescence histograms of cells stained with specific antibody are shown in Fig. 5. There was a bimodal expression of HEA-125 by the type II pneumocytes; this must be considered during interpretation of the median fluorescence values quoted in Table 2. Class I MHC molecules were uniformly expressed by the type II pneumocytes. It was interesting that TX1 showed the highest median expression of HLA-DR; it was subsequently found that this lung was infected. ICAM-1 was constitutively expressed at low levels by all of the cells; median fluorescence values were on average three times higher than control values.

Immunocytochemical analysis of frozen sections of lung (TX1, TX2, TX3) revealed that HEA-125, cytokeratin, class I MHC antigens and the class II antigens HLA-DR and HLA-DP were expressed by alveolar epithelium in situ (Table 3, Fig. 6). HLA-DQ antigens were not seen on the alveolar epithelium on frozen sections, although alveolar macrophages in the frozen lung sections (TX1, TX2, TX3) did express this molecule. ICAM-1 was only observed on frozen sections from two of the three specimens examined during the present study. However, this was an unusual observation, since ICAM-1 expression was always seen on alveolar epithelium in a separate study of 10 normal lung specimens (data not shown). The adhesion molecule B7 was observed on specimens from TX1, VCAM-1 was observed on TX2 and LFA-3 was constitutively expressed by alveolar epithelium in the three specimens examined.

Table 2. Surface antigen expression by isolated type II pneumocytes

	Median fluorescence of isolated type II pneumocytes					
Antibody treatment	Specimen TX1	Specimen TX2	Specimen TX3	Specimen L1	Specimen L2	Specimen L3
Control	5	8	16	11	7	7
HEA-125	563	200	236	287	80	117
Class I MHC (HLA-A,B,C)	204	84	81	272	271	411
HLA-DR	850	210	76	415	178	119
HLA-DP	ND*	86	84	487	882	37
HLA-DQ	ND	12	13	15	81	24
B7	5	7	8	9	15	16
ICAM-1	29	19	20	19	19	47
VCAM-1	5	7	7	7	6	13
LFA-3	7	11	6	9	10	36

*Not determined.

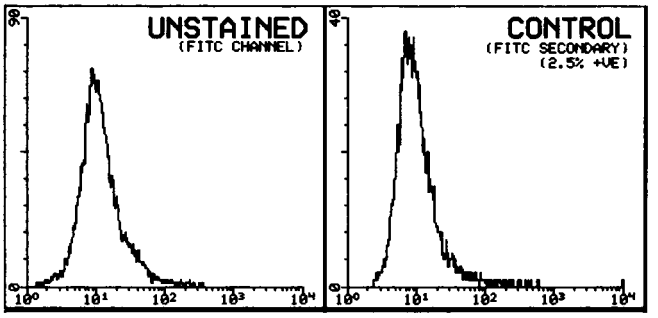

Fig. 4. Control histograms showing unstained cell preparations and cells stained with a negative control IgG + GAMFITC-conjugated antibody.

DISCUSSION

Superior methods to isolate highly purified human type II pneumocytes have been developed. The isolated type II pneumocytes were characterised by the presence of intracellular alkaline phosphatase and lamellar bodies. Carbonyl iron effectively depletes macrophages (Lee, 1980), and was shown to be very efficient in removing alveolar macrophages from the preparation of the type II pneumocytes. The viability of the pneumocytes was not compromised by storing the lung tissue up to 27 hours in Euro-Collin's solution at 4˚C prior to the

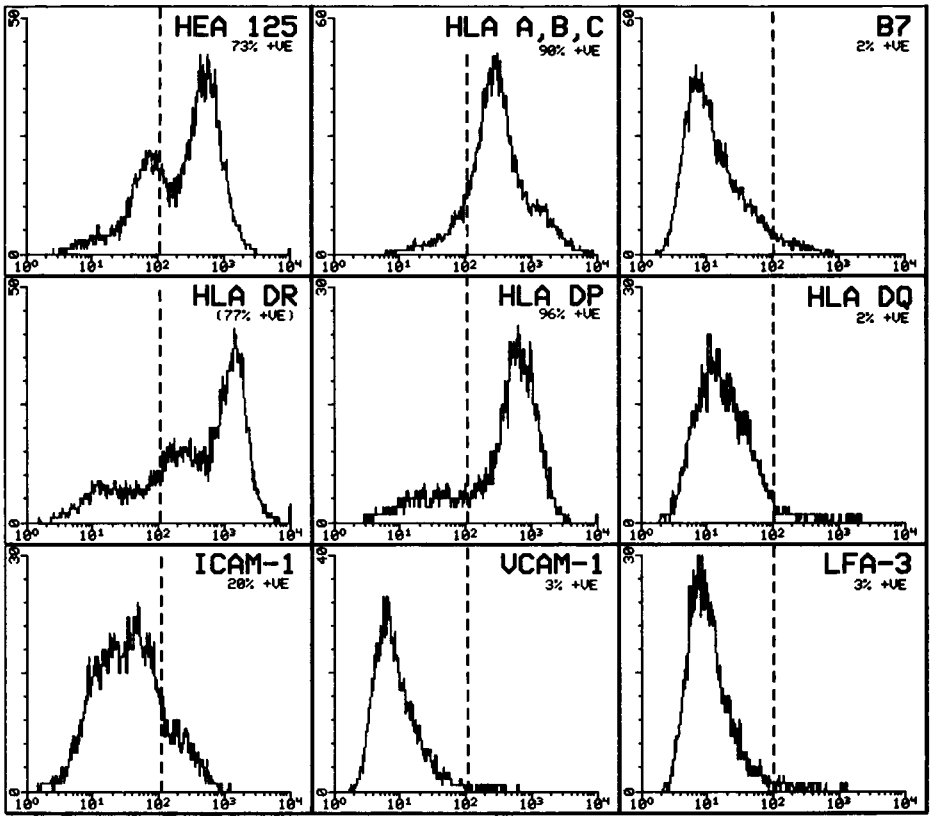

Fig. 5. Representative fluorescence histograms showing the expression of HEA-125, MHC class I, class II and adhesion molecules of isolated type II cells; 3% of the cells stained with the negative control antibody fall to the right of the broken line.

preparation, or by cryopreservation of the purified cells in liquid nitrogen. The electron micrographs demonstrated that the isolated cells were intact and retained good morphology (Fig. 3A,B). Trypsin (0.5%) was found to yield more type II pneumocytes than elastase (30 units/ml). An isolated observation of lower antigen expression of class I and HLA-DR was made on the type II pneumocytes isolated with elastase (TX3) as compared to the five cell preparations isolated with trypsin.

The expression of cell surface antigens on the sections of frozen tissue correlated well with that seen on isolated type II pneumocytes, indicating that protease treatment had not adversely affected the isolated cells. The finding of high levels of HLA-DR expression by type II pneumocytes confirms earlier studies in rat and man. The demonstration of HLA-DP antigen expression is a novel finding. The minimal expression of HLA-DQ antigens is in agreement with a previous report (Glanville et al., 1989). However, in this study lung tissue from healthy, young adults was studied; these specimens may model the normal situation more closely than specimens from elderly lobectomy patients (Glanville et al., 1989) or from fetal/infant lung tissue (Peters et al., 1990). The fact that the highest expression of class II MHC was detected in a specimen of lung tissue subsequently found to be infected (TX1) is of interest, since it has been reported that γ-IFN and inflammatory processes can upregulate class II expression (Schneeberger et al., 1986; Harbeck et al., 1988; Nakayama et al., 1992; Struhar et al., 1989, 1990).

Isolated type II pneumocytes expressed the intercellular adhesion molecule ICAM-1. This finding is in contrast to previous studies of rat type II pneumocytes where no expression of ICAM-1 was detected on primary isolation; however, ICAM-1 was detected when these cells were allowed to differentiate into 'type I' cells in culture (Christensen et al., 1993a,b; Burns et al., 1991). This may reflect species differences or the lower sensitivity of immunofluorescence microscopy and western blot analysis as compared to flow cytometry.

This is the first investigation of the expression of the adhesion molecules VCAM-1, LFA-3 and B7 by alveolar epithelium. These molecules were not detected on the surface of the isolated human type II pneumocytes in this study.

Table 3. Antigen expression by alveolar epithelial cells in frozen sections of lung compared with flow cytometric quantification of expression on isolated type II pneumocytes

	Specimen TX1		Specimen TX2		Specimen TX3	
Antibody treatment	Tissue section*	Flow cytometry†	Tissue section	Flow cytometry	Tissue section	Flow cytometry
HEA-125	+	+++	+	+++	+	+++
Class I MHC (HLA-A,B,C)	+	+++	+	++	+	++
HLA-DR	+	+++	+	+++	+	++
HLA-DP	+	ND‡	+	++	+	++
HLA-DQ	–	ND	–	–	–	–
B7	+	–	–	–	–	–
ICAM-1	+	+	+	+	–	+
VCAM-1	–	–	+	–	–	–
LFA-3	+	–	+	–	+	–

*Graded as present (+) or absent (–) on epithelium.
†Quantitatively graded from present at high levels (+++) to absent (–).
‡Not determined.

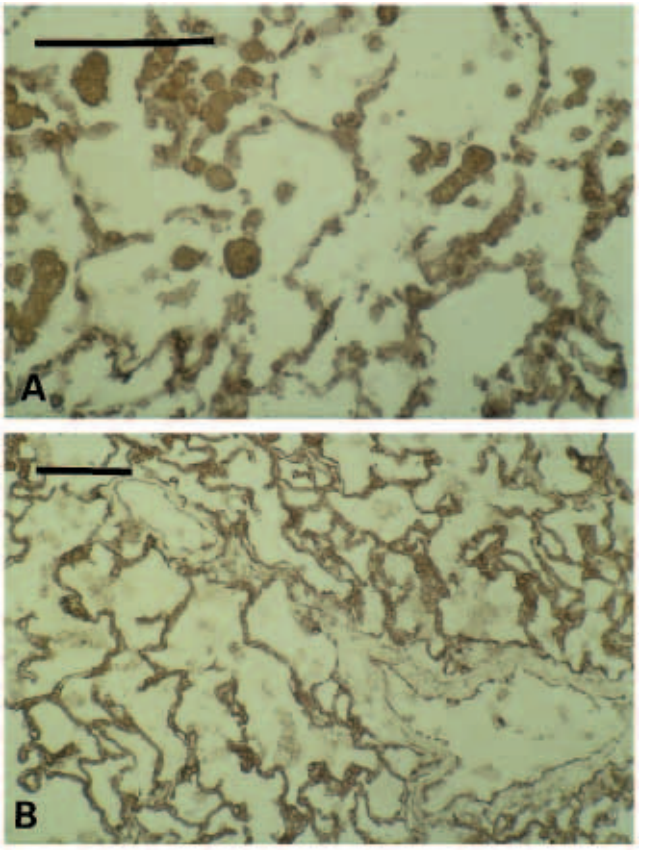

Fig. 6. Immunohistochemical staining of normal lung with (A) HLA-DR and (B) ICAM-1. Bar, 50 μm.

VCAM-1 was uniformly expressed on the vessels of the frozen sections of lung specimens and was observed on the alveolar tissue in one of the three specimens (TX2). LFA-3 expression was observed in the alveolar epithelium on frozen sections but not isolated type II cells, suggesting that LFA-3 was only expressed by type I pneumocytes, which were not distinguishable from II pneumocytes in these sections.

In conclusion, a reproducible method for the isolation of pure populations of human type II pneumocytes has been developed. These cells were not damaged by the isolation procedure. Work is in progress to define the role type II pneumocytes play in local regulation of the pulmonary immune response.

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