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ORIGINAL ARTICLE

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In situ characterization of human cytomegalovirus infection of bronchiolar cells in human transplanted lung

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Abstract Distal airway cell infection by human cytomegalovirus (HCMV) in transplanted lung has been occasionally reported but not systematically investigated. The present study aimed at testing the prevalence of HCMV bronchiolar infection in human transplanted lung. We identified and immunophenotyped, with double labeling, infected lung cells in 31 transbronchial biopsies with HCMV infection, containing distal airways (7 HCMV pneumonias, 7 HCMV infection without inflammation, and 17 morphologically occult, non-cytopathic HCMV infection). HCMV-infected cells in pneumonias, localizations, and occult infections were alveolar epithelia (32.8%, 42.8%, and 53.5%, respectively), endothelia (22.9%, 24.7%, and 26.4%, respectively), macrophages (0.006%, none, and none, respectively), airway epithelia (0.01%, 8.9%, and none, respectively), and bronchiolar smooth muscle cells (0.011%, 14.6%, and 16.1%, respectively). Ciliated and bronchiolar smooth muscle cells in transplanted lung only occasionally harbored viral infection and never showed viral cytopathy. On the basis of our morphological observations, HCMV infection of bronchiolar wall cells is rare, while alveolar epithelia and capillary endothelial cells are the major targets of lung infection.

Keywords Human cytomegalovirus · Lung transplantation · Bronchiolar epithelial cells · Smooth muscle cells

Introduction

Human cytomegalovirus (HCMV) opportunistic infection frequently affects transplanted lungs [6, 9]. The characteristics of viral infection in the different lung cells are still poorly defined; virus may enter the cell through receptor-mediated mechanisms in targeted cells or as a result of phagocytic activity [23, 26, 31]. Once infected, cells such as endothelia and alveolar epithelia can be fully permissive for viral replication and develop viral cytopathy, which morphologically characterizes the late stage of viral replication [30], or may host latent infection (monocyte/macrophages) and become permissive only when specifically activated [17, 34]. Finally, infected cells, such as polymorphonuclear granulocytes, may be simple carriers of viral particles, antigens, and DNA [26].

Most knowledge on HCMV lung infection derived from studies in human immunodeficiency virus (HIV)-positive patients and focused on pneumonia and endothelialitis, while bronchiolar infection has only been occasionally reported [18, 21, 22, 30, 35, 38]. In particular, little is known about HCMV infection of airway cells in transplanted lung.

The present study aimed at identifying and quantifying, on a morphological and immunohistochemical basis, HCMV-infected cell populations in a consecutive series of transbronchial biopsy samples in order to provide in vivo and in situ data of bronchiolar viral tropism.

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Materials and methods

Patients and biopsy series

We characterized, with histopathologic and double immunostain techniques, early and late HCMV-infected cells in a consecutive series of 186 transbronchial biopsies (TBBs), in which light microscopy examination identified either terminal bronchi or bronchioles. TBBs were obtained from 65 patients (mean age 41.9±12.7 years, 41 males) who underwent lung transplantation and retransplantation (n=3) at our hospital over the last 5 years. Of the patients, 18 received a heart and lungs, 21 received both lungs, and 29 received a single lung. The immunosuppressive protocol was based on a triple combination of cyclosporin, azathioprine, and steroids, supplemented with an initial 7-day course of antilymphocyte globulins [3].

Table 1 Antibodies used for the characterization of human cytomegalovirus (HCMV) infectable lung cell populations. *EMA* epithelial membrane antigen

Antibodies	Source	Specificity	Cell population	Dilution
QBEND/10	Serotec (Oxon, UK)	Human antigen CD34	Endothelia	1:100
E29	Dako (Glostrup, Denmark)	Human EMA	Alveolar epithelial cells, occasional bronchial epithelia	1:100
PGM1	Dako	Human antigen CD68	Tissue monocytes and macrophages	1:80
AE3	Biogenex (San Ramon, Calif.)	High molecular weight cytokeratins	Bronchial, bronchiolar, and activated alveolar epithelial cells	1:5
Cathepsin D	Triton Diagnostic (Alameda, Calif.)	Cathepsin D (34 kDa and 48 kDa)	Bronchial epithelial cells; alveolar macrophages	1:100
HHF35	Enzo Biochemicals (Farmingdale, N.Y.)	Alpha and gamma isotypes of skeletal, cardiac, and smooth muscle actin	Smooth muscle cells	1:100

TBB sampling and processing

Each biopsy procedure yielded 4–9 lung tissue samples. Sampling via fiberoptic bronchoscope was selectively guided by abnormalities seen at lung roentgenography; random samples were taken from roentgenographically normal lungs or lobes. TBB samples were fixed in formalin for 20 min, processed with a previously described procedure that optimally preserves most relevant antigens for immunohistochemical reactions [2], and then embedded in paraffin. One hundred and twenty sections (4-µm thick) were cut from each paraffin block and collected on 40 slides. Paraffin sections were stained with hematoxylin and eosin (HE) and with Movat pentachrome for the histologic diagnosis of acute and chronic rejection and other transplantation-related complications. Unstained paraffin sections were used for immunohistochemical reactions.

Diagnosis of acute and chronic lung rejection

Lung rejection was diagnosed in accordance with the criteria proposed by the International Society for Heart and Lung Transplantation [40].

Histopathologic and immunohistochemical detection of HCMV lung infection

Conventional histopathologic diagnosis of HCMV infection and of related disease was based on well-recognized and generally agreed upon morphological criteria. Viral cytopathy with nuclear inclusions was the marker that defined overt HCMV infection. HCMV infection was called pneumonia when associated with inflammatory infiltrates and localization when cytopathic cells were found in areas free from infiltrates. HCMV-infected cells were identified with a monoclonal antibody (BS500, Biotest, Dreieich, Germany) that recognizes an epitope contained within the HCMV immediate-early (IE) 1-pp72 [2, 25]. The antibody does not detect HCMV-related herpes virus antigens (Epstein-Barr virus and herpes virus 1) in cultured control cells. The antibody immunostained the megalic nuclei of HCMV-infected cytopathic cells and also normal-sized nuclei of non-cytopathic-infected cells. HCMV infection was defined as occult when viral cytopathy was absent, and non-megalic-infected cells were exclusively identified by the immunostaining of their nuclei with the specific HCMV IE antigen antibody [2].

Immunohistochemical phenotyping of HCMV-infected lung cells

For immunohistochemical characterization of HCMV-infected cell populations, we adopted a double-immunostaining technique. Slides were sequentially incubated with anti-HCMV BS500 antibody and with antibodies to lung cell populations (Table 1). Two non-contiguous slides were stained with each marker. HCMV immunostaining was performed with the avidin-biotin-peroxidase complex (Dako, Glostrup, Denmark), using diaminobenzidine tetrahydrochloride as the chromogen substrate (brown nuclear stain). The second reaction was revealed with streptavidin-conjugated alkaline phosphatase (Biogenex, San Ramon, Calif.) and with fast red as the chromogen (red cytoplasmic and/or membrane stain). Slides were weakly counterstained with Harris hematoxylin. PGM1 and AE3 immunostain was preceded by a 10-min enzymatic predigestion with trypsin (0.05% in 0.15 M Tris buffer, pH 7.6, containing 0.05% $CaCl_2$) at 37°C. To immunocharacterize BS500-positive, PGM1-reactive macrophages, 4-chloro-1-naphtol was used as the chromogen substrate for the BS500 immunoreaction (brown–black nuclear stain), to avoid non-specific reactions of diaminobenzidine with the intracytoplasmic inclusions of alveolar macrophages. Nuclear counterstain was omitted.

Analysis

Immunohistochemical reactions were analyzed using light microscopy (Zeiss Axioplan). For each TBB, six different sections on two slides were examined for each marker. The total number of BS500-positive cells and the number of double-positive cells (BS500+marker positive cells) were counted. The percentage of BS500-positive cells immunotyped with each different antibody was calculated on the overall number of BS500-positive cells in slides stained with the given antibody.

Controls

Negative controls consisted of lung tissue sampled from HCMV-negative organ donors not suitable for lung transplantation. HCMV-infected human embryonic lung fibroblast (HELF) cell cultures and lung autopsy samples from acquired immunodeficiency syndrome (AIDS) patients with clinically documented and pathologically proven HCMV pneumonia were used as positive controls for HCMV detection. The above lung samples were also employed as positive controls for markers of lung cell populations. Single steps of the immunoreaction were omitted for negative controls of all immunohistochemical reactions.

Limits of the study

The major limit of the study was that counting and immunophenotyping of identical pools of infected cells was prevented in the different doubly immunostained tissue sections. Since sections were

Table 2 Number of BS500-positive, human cytomegalovirus (HCMV)-infected cells and number of BS500-positive cells that immunoreacted with specific markers for lung cell populations. *EMA* epithelial membrane antigen; *CK* cytokeratin

Overall number of BS500-positive cells[a]	Lung cell marker	Number of double-positive cells	Percentage
Pneumonia (*n*=7)			
593	CD68 (macrophages)	4	0.006
589	CD34 (endothelia)	135	22.9
884	EMA (alveolar epithelia)	290	32.8
638	CK/cathepsin D (respiratory epithelia)	7	0.01
686	Actin (smooth muscle cells)	8	0.011
Localization (*n*=7)			
157	CD68 (macrophages)	0	0
206	CD34 (endothelia)	51	24.7
175	EMA (alveolar epithelia)	76	42.8
201	CK/cathepsin D (respiratory epithelia)	18	8.9
260	Actin (smooth muscle cells)	38	14.6
Occult infection (*n*=17)			
36	CD68 (macrophages)	0	0
34	CD34 (endothelia)	9	26.4
28	EMA (alveolar epithelia)	15	53.5
29	CK/cathepsin D (respiratory epithelia)	0	0
31	Actin (smooth muscle cells)	5	16.1

[a] The overall number of BS500-positive cells is different within the same groups (pneumonia, localization, and occult infection), because their number varied from section to section. The total number of double-positive cells and relative percentage is referred to as the overall number of BS500-positive cells in sections stained with the specific antibody directed against each cell population. For this reason, the overall percentage of double-positive cells in each group does not add up to 100%. The number of double-stained sections is identical for each marker (*n*=6)

cut at 4-µm intervals, single BS500-positive nuclei, that had a 4- to 8-µm diameter, could be seen only on a few consecutive sections. Given the proven absence of HCMV replication in human polymorphonuclear granulocytes and in lymphocytes [10, 26], double immunostain with anti-HCMV antigens and with markers specific for these cells was not performed.

Results

Overt and occult lung infections

HCMV overt cytopathic infection was identified in 14 biopsies (7.4%); pneumonia (lung cytopathic cells surrounded by inflammatory cells) in 7 and localization (cytopathic cells not surrounded by inflammatory infiltrates) in 7. HCMV occult infection (nuclear viral antigens and absence of cytopathy) was identified in 17 biopsies (9%). In 9 of them, acute rejection-like infiltrates were close to the HCMV BS500 antigen-positive cells. In 8, positive cells were found in areas free from acute rejection.

BS500 specifically stained intranuclear viral antigens of morphologically proven cytopathic and of non-cytopathic cells, both in control cells and tissues, and in the study samples. In biopsies with overt infection, both pneumonia and localization, all megalic nuclei immunoreacted with BS500 antibody; several non-cytopathic, BS500-positive nuclei were also observed. The number of BS500-positive cells was higher in pneumonia (48.4 cells/biopsy slide) than in localizations (14.2 cells per biopsy slide) and in occult infections (0.9 cells per biopsy slide).

HCMV infection in bronchial and bronchiolar epithelial cells

The data obtained from HCMV infection in bronchial and bronchiolar epithelial cells is presented in Table 2. Infected bronchial and bronchiolar epithelia were identified with immunostain using high molecular weight cytokeratins and cathepsin D antibodies and on the basis of their morphology and tissue location, since none of the tested antibodies was specific for ciliated cells (cytokeratin stain was occasionally observed in activated alveolar epithelia). Doubly positive cells were observed in four biopsies (14% of HCMV-positive TBBs), one pneumonia and three localizations. Their numbers were extremely low, from one to eight per biopsy. In pneumonia and in localization, 0.01% and 8.9% of the HCMV-infected cells, respectively, were bronchial and bronchiolar epithelia. In three biopsies, positive basal cells were part of small chains of desquamated bronchial epithelia (Fig. 1a, c, and e). Few BS500-positive epithelia of a cartilaginous bronchus were seen in only one sample infiltrated by mixed (polymorphonuclear granulocytes, lymphocytes, and plasma cells) inflammatory cells (Fig. 1b).

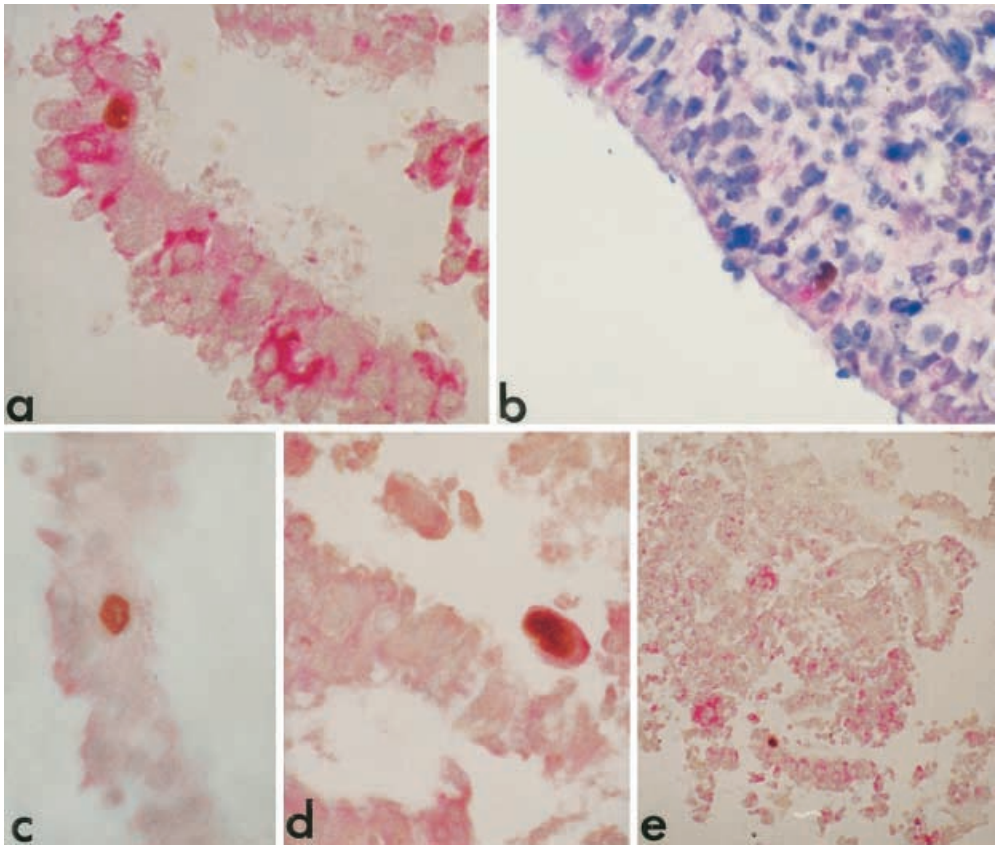

Fig. 1 Light micrographs showing double immunostaining with BS500 and AE3 anti-high molecular weight cytokeratins (**a**, **d**, and **e**) and anti-cathepsin D (**b**) and anti-epithelial membrane antigen (EMA; **c**) antibodies. **a** Human cytomegalovirus (HCMV)-infected bronchial epithelial cell in a chain of cytokeratin-immunoreactive epithelia detached from the bronchial wall. **b** HCMV-infected, cathepsin D-immunoreactive ciliated cell in a bronchiolar epithelium with severe intraepithelial and subepithelial inflammation. **c** Single HCMV-infected, ciliated bronchial cell in a small chain of detached epithelia, faintly stained with EMA. **d** Single cytokeratin-positive, HCMV-infected columnar cell. **e** Low-power view of detached, cytokeratin-immunoreactive bronchial epithelia with a single HCMV-infected cell. **a**–**e** Immunoreactions revealed with diaminobenzidine (BS500) and fast red (AE3, EMA, cathepsin D); Harris hematoxylin nuclear counterstain

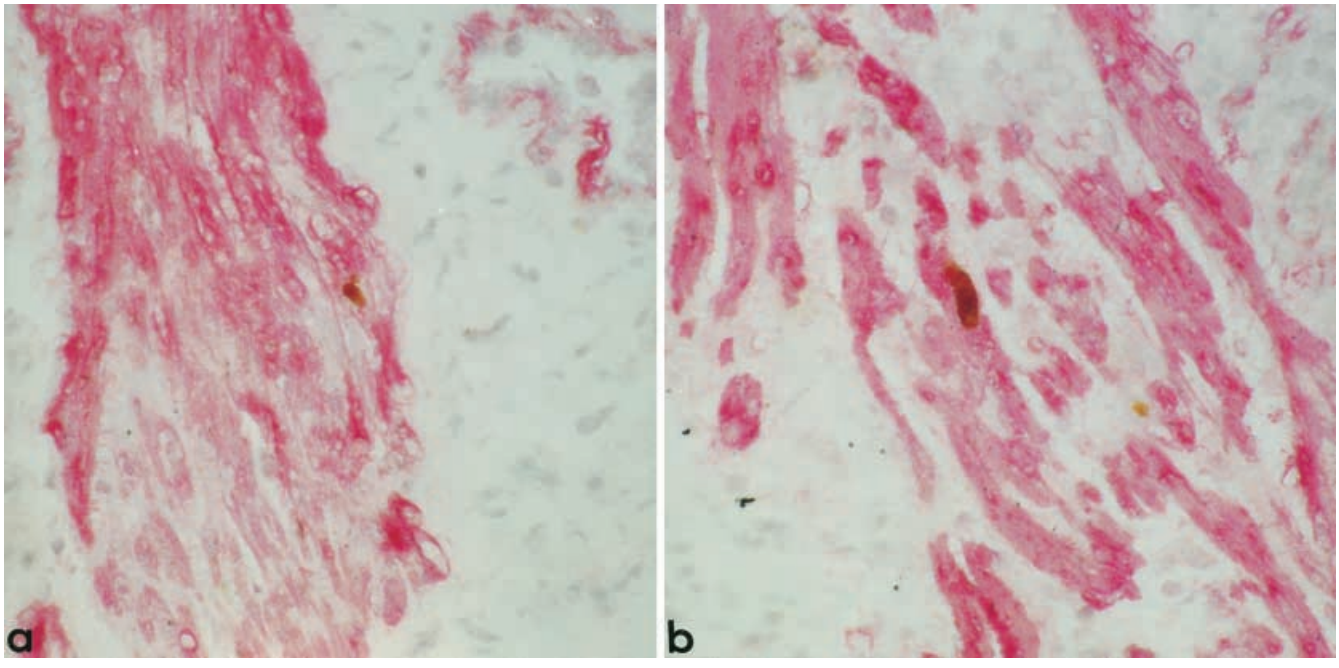

Fig. 2 Light micrographs showing double immunostain with BS500 and HHF35 anti-actin antibodies in peribronchial smooth muscle cell bundles from a single transbronchial biopsy with normal-sized (**a**) and moderately enlarged (**b**) nuclei. **a**, **b** Immunoreactions were revealed with diaminobenzidine (BS500) and fast red (HHF35); Harris hematoxylin nuclear counterstain

HCMV infection in smooth muscle cells

The data obtained from HCMV infection in smooth muscle cells is presented in Table 2. Smooth muscle cells in the bronchial and vascular walls were immunophenotyped with an antibody directed against a pool of smooth

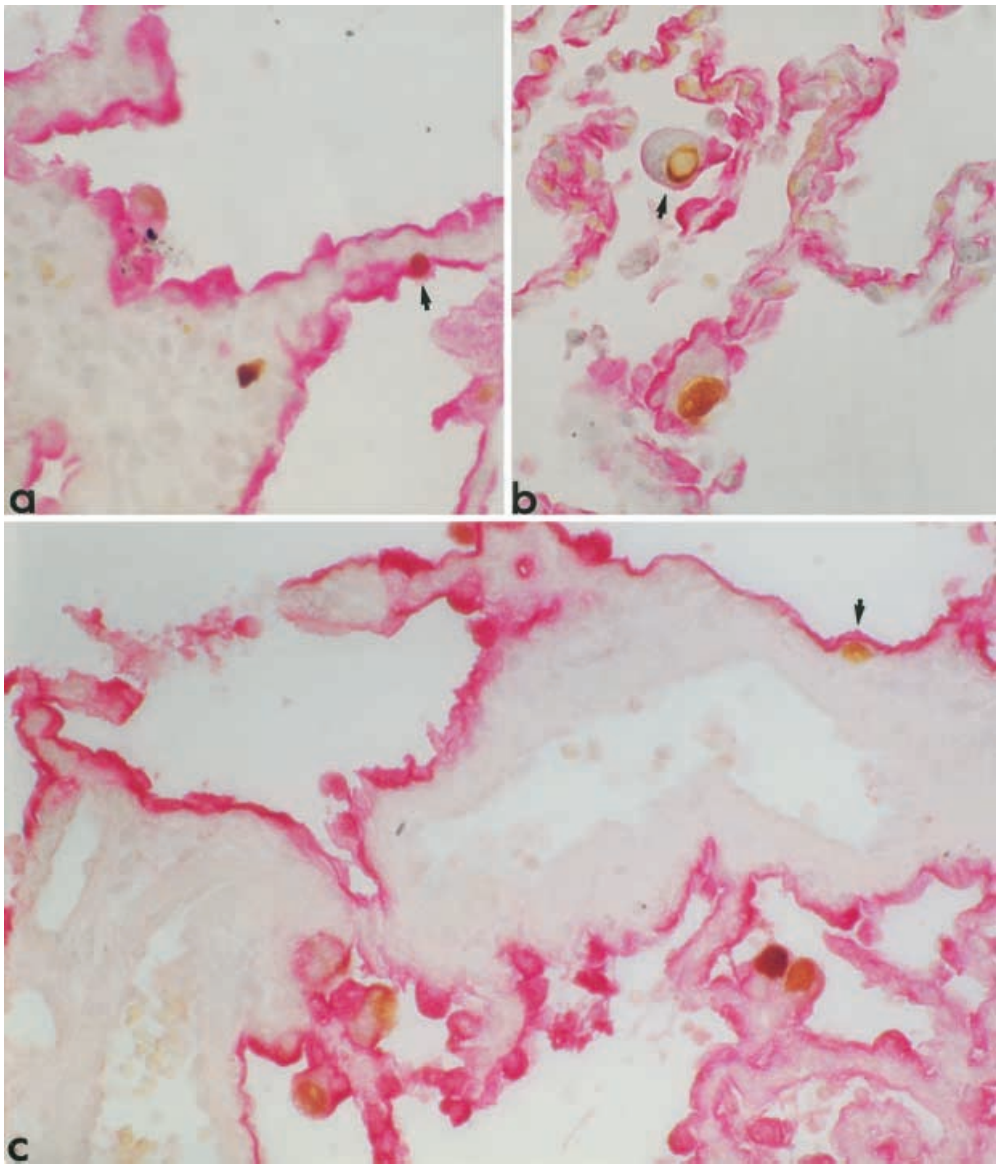

Fig. 3 Light micrographs showing double immunostaining with BS500 and E29 antibodies. **a** Non-cytopathic human cytomegalovirus (HCMV)-infected alveolar epithelial cell (*arrow*). **b** Cytopathic alveolar epithelia show weak epithelial membrane antigen (EMA) immunostain; one epithelial cell (*arrow*) is detached from the alveolar wall and lies free in the alveolar space. **c** Different stages of HCMV infection in EMA-positive alveolar epithelia, from flat, non-cytopathic (*arrow*) to megalic cells protruding in the alveolar spaces. **a**–**c** Immunoreactions were revealed with diaminobenzidine (BS500) and fast red (EMA); Harris hematoxylin nuclear counterstain

and striated muscular actin isoforms (Fig. 2). HCMV infection was identified in smooth muscle cells of both bronchial/bronchiolar walls and arteriolar media. Infected smooth muscle cells were rare. They were seen in seven TBBs, four with HCMV localization, two with pneumonia, and one with occult infection. In pneumonia, in localization, and in occult infection, 0.011%, 14.6%, and 16.1% of the HCMV-infected cells, respectively, were immunophenotyped with actin immunostaining. In all cases, the number of infected cells was low: 1 to 12. None showed overt viral cytopathy. In a biopsy with severe acute bronchial inflammation, the nuclei of some infected peribronchial smooth muscle cells were slightly enlarged, particularly in their lesser diameter (6 µm; Fig. 2b).

HCMV infection in alveolar epithelial cells

The data obtained from HCMV infection in alveolar epithelial cells is presented in Table 2. Alveolar epithelia were the cell population most commonly infected by HCMV. In pneumonia, localization, and occult infection, 32.8%, 42.8%, and 53.5% of the HCMV-infected cells, respectively, were immunophenotyped using epithelial membrane antigen (EMA) immunostaining. HCMV-infected alveolar epithelia had both megalic and normal size nuclei (Fig. 3). Most non-megalic, BS500-positive cells were type-II cuboidal alveolar epithelia. Less frequently, type-I, flat alveolar epithelia were BS500-positive (Fig. 3a, c). Most EMA-positive cells, either megalic or non-megalic, were attached to the alveolar walls, while only rare EMA-positive megalic cells were free in the alveolar spaces (Fig. 3b). Their non-macrophagic nature was further confirmed with negative CD68 PGM1 immunostain.

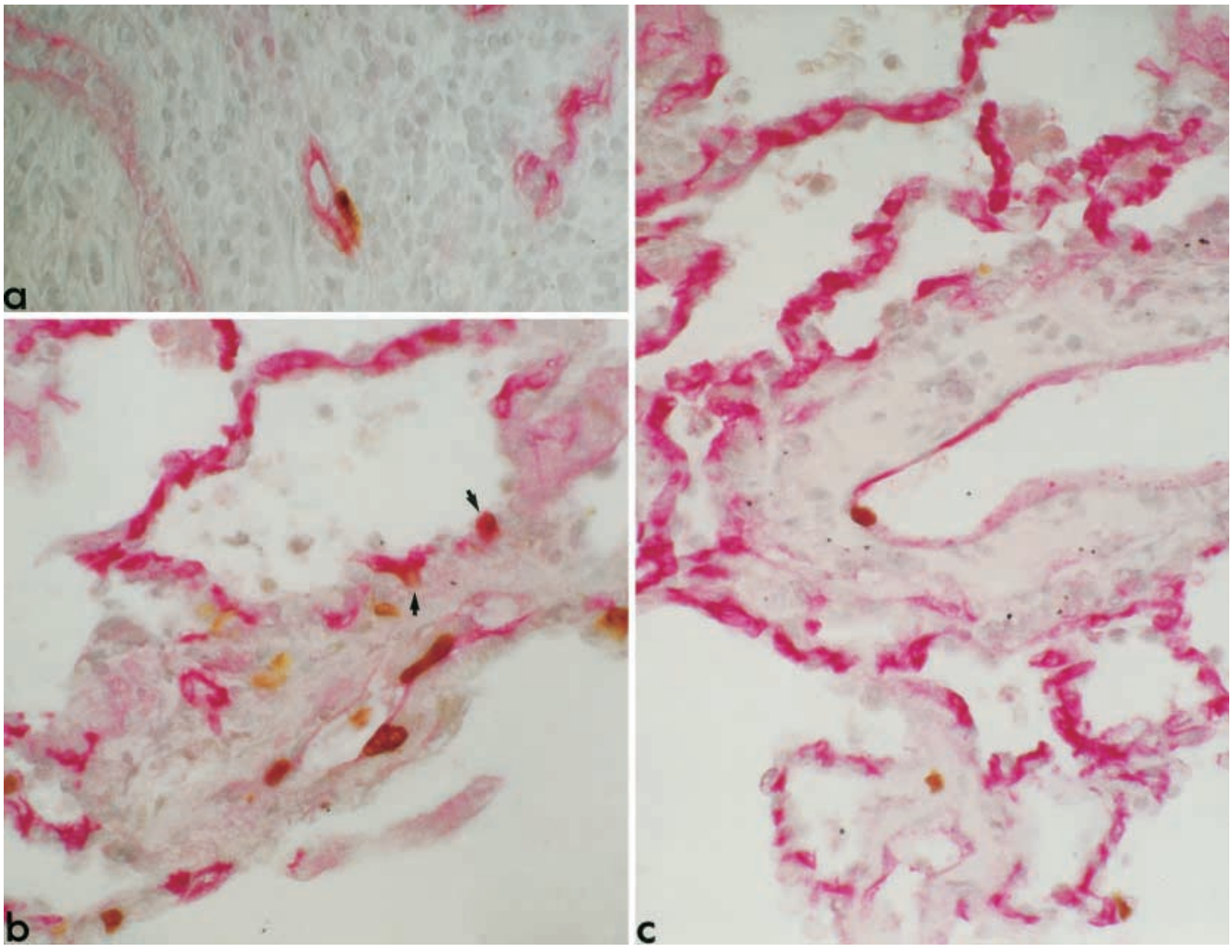

Fig. 4 Light micrographs showing double immunostaining with BS500 and Qbend/10 anti-CD34 antibodies. **a** Human cytomegalovirus (HCMV)-infected endothelial cell from a peribronchiolar capillary vessel. **b** HCMV infection in non-cytopathic endothelia from septal capillary vessels (*arrows*) and in venular endothelia. **c** HCMV infection in a single arteriolar endothelial cell. **a**–**c** Immunoreactions revealed with diaminobenzidine (BS500) and fast red (CD34); Harris hematoxylin nuclear counterstain

HCMV infection in endothelial cells

The data obtained from HCMV infection in endothelial cells is presented in Table 2. Endothelial cells (CD34 positive) were the second most frequent HCMV-infected lung cellular population (22.9%, 24.7%, and 26.4%, respectively; Fig. 4). In two biopsies with epithelial HCMV localization, all endothelial cells were negative, while in three biopsies (one pneumonia and two localizations) most infected cells were endothelia. When referring to the vessel type, HCMV endothelial infection was higher in alveolar wall capillaries than in peribronchiolar capillaries, arterioles, and venules, in that order.

HCMV infection in alveolar and interstitial monocytes/macrophages

The data obtained from HCMV infection in alveolar and interstitial monocytes/macrophages is presented in Table 2. Nuclear infection of cells of the monocyte–macrophage lineage (CD68-PGM1 positive) was rare. PGM1-reactive cells with BS500 nuclear immunostain were observed in two TBBs, both with overt pneumonia; a single positive cell in one biopsy and three cells in the other (Fig. 5). Double PGM1- and BS500-positive cells had little cytoplasm that did not contain digestion residues; none of these cells showed viral cytopathy. The positive cells were free in alveolar spaces.

HCMV infection and obliterative bronchiolitis relationship

Of the overall 186 biopsies, obliterative bronchiolitis (OB) was morphologically proven in 26. One of these had occult HCMV infection, and immunohistochemical study did not show HCMV infection of bronchiolar cells.

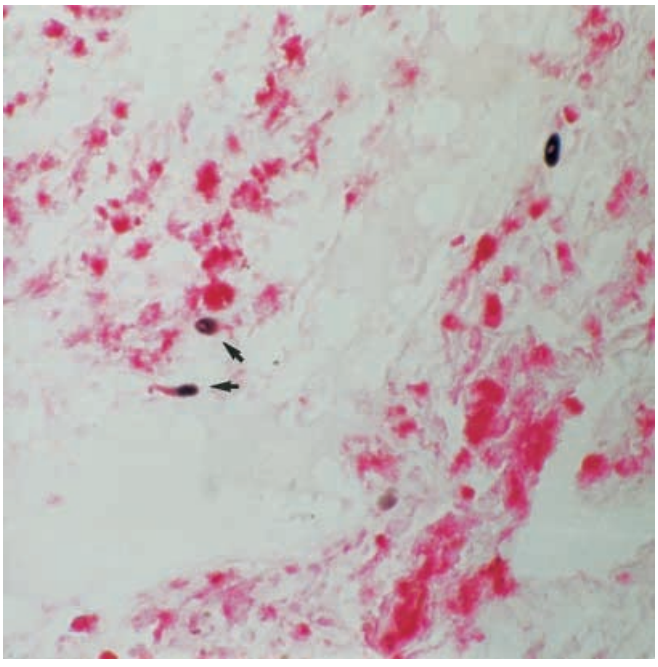

Fig. 5 Light micrographs showing double immunostaining with BS500 and PGM1 anti-CD68 antibodies. Rare PGM1-positive, human cytomegalovirus (HCMV)-infected macrophages observed in a biopsy with HCMV pneumonia (*arrow*). Immunoreactions revealed with diaminobenzidine (BS500) and fast red (PGM1); no nuclear counterstain

Discussion

The present study documents a very low prevalence of HCMV-infected bronchiolar wall cells in human transplanted lung. Cell populations preferentially infected by the virus in transplanted lungs are alveolar epithelial and endothelial cells, followed by smooth muscle, airway ciliated cells, and macrophages, in that order. Similar findings were reported in native lungs from non-transplant, immunocompromised patients with systemic HCMV infections [14, 21, 22, 27, 30, 35, 39] and, on one occasion, in transplanted lungs [38]. In transplanted lung, both alveolar epithelia and endothelia are known to be fully permissive to viral replication and to host latent and occult infection [2], while inflammatory cell infection is limited to observation of occult infection in occasional macrophages [22, 30]. Knowledge of HCMV infection in other lung cell populations is still limited.

Based on the present results, epithelial airway cells do not seem to be an elective site for HCMV infection in transplanted lungs. In particular, in none of our cases was the infection observed in the epithelial cells of terminal bronchioli. The practical application of our data is that the occurrence of HCMV bronchiolitis in transplanted lungs is extremely unlikely. This observation fits with the occasional HCMV bronchiolitis described in a few non-transplanted patients [18, 36] and with the absence of reports on HCMV bronchiolitis in a transplant setting.

The tropism of HCMV for smooth and striated heart muscle is known and documented [1, 21]. Prior observations of viral DNA and antigens in rare smooth muscle cells from the airway walls [30] and from the media of atherosclerotic vessels [24] support our observation on smooth muscle cells from bronchiolar walls and from lung vessel media. Overt cytopathy was not observed in HCMV-infected smooth muscle cells from our transplanted lungs. However, few permissive smooth muscle cells were elegantly shown in acute cytomegalovirus gastritis in a kidney transplanted patient [29]. Although rare, infected airway smooth muscle cells could represent an elective site of HCMV latency and immune surveillance escape.

The reported link between HCMV infection and lung chronic rejection relies on the significantly higher prevalence of OB in patients with HCMV pneumonia [7, 11, 13, 15, 32]. Whether HCMV plays a direct or indirect role in OB has not been elucidated. Our results show that infection of bronchiolar cells is occasional and suggest that the likelihood of a causal role of their infection in OB development is low. However, it can not be excluded that latent or occult HCMV infection in smooth muscle cells and myofibroblasts of the bronchiolar wall may trigger their activation, proliferation, and synthetic function, as hypothesized for restenosis in atherosclerotic vessels [8]. An indirect role of HCMV infection in the development of chronic bronchiolar lung rejection is more likely. Distal airways seem to be sensitive to persistent immune-mediated injury [28]. In HCMV pneumonia, the infection-related lymphomonocytic inflammation persistently enhances allograft reactivity through the cytokine-mediated expression of both adhesion and major histocompatibility (MHC) class-II molecules in epithelia and endothelia, as shown in animal models [33] and by concomitant activation of various leukocyte populations [12]. Areactive HCMV infection has been suggested to increase donor-specific alloreactivity [13] and to negatively affect airway function through immune-mediated mechanisms [7, 13, 16, 32]. In in vitro models, adhesion molecule expression and T-lymphocyte activation (but not MHC class II expression) are directly induced by HCMV in infected cells [4, 20, 37]. Furthermore, HCMV immediate-early genes induce transcriptional activation of cytokine-coding genes in infected fibroblasts and in peripheral blood leukocytes [5, 19]. However, in human transplanted lungs, the overall number of cells harboring viral antigens, both in occult infections and in viral localizations, seems too low to induce a locally relevant immunological activation.

In conclusion, we have found that HCMV infection and IE antigen expression is exceedingly more frequent in alveolar epithelial and endothelial cells than in ciliated epithelia, in bronchiolar smooth muscle cells, and in macrophages. Therefore, HCMV bronchiolitis caused by viral infection of bronchiolar cells is unlikely to occur in transplanted lungs.

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