Pediatric AIDS-Associated Lymphocytic Interstitial Pneumonia and Pulmonary Arterio-Occlusive Disease

Role of VCAM-1/VLA-4 Adhesion Pathway and Human Herpesviruses

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Because the mechanisms of lymphocyte accumulation in the lungs of children with AIDS-associated lymphocytic interstitial pneumonia (LIP) are unknown, we studied the relative contributions of known adhesion pathways in mediating lymphocyte adherence to endothelium and the potential role of human herpesviruses in the expansion of these lesions. LIP was characterized by lymphoid hyperplasia of the bronchus-associated lymphoid tissue (BALT) and infiltration of the pulmonary interstitium with CD8⁺ T lymphocytes. In some individuals there was expansion of the alveolar septae with dense aggregates of B lymphocytes, many containing the Epstein-Barr viral (EBV) genome. Patients with concurrent EBV infection also demonstrated large-vessel arteriopathy characterized by thickening of the intima with collagen and smooth muscle. Venular endothelium from the lung of children with LIP, but not uninflamed lung from other children with AIDS or lung from children with nonspecific pneumonitis, expressed high levels of vascular cell adhesion molecule-1 (VCAM-1) protein. In turn, inflammatory cells expressing very late activation antigen-4 (VLA-4), the leukocyte ligand for VCAM-1, were the predominant perivascular infiltrate associated with vessels expressing VCAM-1. Expression of other endothelial adhesion molecules, including intracellular adhesion molecule-1 and E-selectin, was not uniformly associated with LIP. Using a tissue adhesion assay combined with immunohistochemistry for VCAM-1, we show

that CD8⁺ T cell clones that express VLA-4 bind preferentially to pulmonary vessels in sites of LIP: vessels that expressed high levels of VCAM-1. When tissues and cells were pretreated with antibodies to VCAM-1 or VLA-4, respectively, adhesion was inhibited by ≥80%. Thus, infiltration of alveolar septae with CD8⁺ T cells was highly correlative with VCAM-1/VLA-4 adhesive interactions, and focal expansion of B cells was coincidental to co-infection with EBV. (*Am J Pathol* 1999, 154:1453–1464)

The high death rate seen in children infected with human immunodeficiency virus type 1 (HIV) at birth and during the first year of life is most often attributed to pneumonia caused by opportunistic infections.^{1,2} After the first year, the most frequently seen pulmonary complications are chronic lymphoid processes.^{3–6} Lymphocytic interstitial pneumonia (LIP) is recognized as an AIDS-defining diagnosis in children under 13, as specified by the Centers for Disease Control and Prevention (CDC) pediatric AIDS case definition.⁷ Because children with LIP generally have a longer average survival time than those who acquire opportunistic pulmonary infections,⁶ this distinction is important for prognosis and treatment. Still, little is known regarding the pathogenesis of LIP.

Aggressive B-cell non-Hodgkin lymphomas and fatal polyclonal lymphoproliferative disorders occur with increased frequency among children with HIV AIDS.^{5,8–11} Many of these neoplastic and pre-neoplastic conditions have been attributed to co-infection with human herpesviruses.^{12–15} Interactions between herpesviruses and HIV have been demonstrated *in vitro* through experiments showing transactivation, CD4 up-regulation, Fc receptor

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Table 1. Histomorphological and Immunohistochemical Characteristics of Inflammatory and Vascular Lesions in Children with AIDS-Associated Lymphocytic Interstitial Pneumonia

Case	LIP	Mφ ISH/IHC	VCAM-1	Arteriopathy		
				Vasculitis	Intimal fibrosis	Fibrosis and/or calcification of media
1	+++*†	++++/++++	8	+	+	—
2	+++*	++++/++++	6	+	+	—
3	++	++/+	6	—	—	—
4	++†	+/+	2	—	—	—
5	++	+/-	3	—	—	—
6	+++*	++++/++++	8	+	+	+

Specimens were obtained from W. Travis, National Institutes of Health, Bethesda, MD, and S. Brodie, Department of Laboratory Medicine, University of Washington, Seattle, WA.

LIP was scored as follows: +, multifocal interstitial leukocytes; ++, multifocal and intermittent confluent areas of leukocyte infiltration; +++, confluent areas of alveolar septal thickening with leukocytes and lymphocyte aggregates with or without germinal centers. ISH/IHC, *in situ* hybridization for HIV mRNA/immunohistochemistry for HIV gag p24 protein. Results represent an average of 10 microscopic fields (10×) as follows: —, no positive cells; +, 1 to 5 positive cells; ++, 5 to 10 positive cells; +++, 10 to 15 positive cells; +++, greater than 15 positive cells. For VCAM-1, immunoreactive vessels were assigned scores as follows: 0, no vessels; 1, 1 to 4 vessels; 2, 5 to 10 vessels; 3, 11 to 15 vessels; 4, 16 to 20 vessels; 5, >20 vessels. Staining intensity was scored as follows: 0, no reactivity; 1, faint; 2, moderate, with or without 4 vessels being intense; 3, intense staining of >4 pulmonary vessels. The sum of these two values was then used as the final score, which had a theoretical range from 0 to 8. Arteriopathy was characterized by special stains for inflammation within the vascular wall (vasculitis), intimal fibrosis, fragmentation of elastic tissue and/or fibrosis and calcification of media. These categories were scored as positive (+) or negative (—).

* Concurrent EBV.

† Concurrent KSHV.

‡ Concurrent human CMV.

induction, pseudotype formation, cytokine production, and antigen presentation.^{16–18} Still, the role of human herpesviruses in LIP, strictly as opportunists or as co-factors in HIV disease, is yet unknown.

Cell-to-cell and cell-to-extracellular matrix interactions are important in cell migration. The binding of lymphocytes to vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and/or intracellular adhesion molecule-1 (ICAM-1) expressed on vascular endothelial cells accounts for a significant portion of the binding of lymphocytes to endothelium.^{19–22} Collectively, these molecules differ in their leukocyte binding repertoire. The integrin $\alpha 4\beta 1$ that defines very late activation antigen-4 (VLA-4), is the leukocyte ligand for VCAM-1 and is expressed by a restricted set of cell types, including monocytes^{21,23} and lymphocytes,^{22,24,25} and is present at low levels on some polymorphonuclear cells.^{26,27} E-selectin also mediates the adhesion of polymorphonuclear cells,²⁸ monocytes,²⁹ and certain lymphocyte subsets.^{19,30} In contrast, ICAM-1 promotes the adhesion of all leukocytes that bear the surface receptor leukocyte function-associated antigen-1 (LFA-1).^{31,32}

The release of chemotaxins in concert with the expression and avidity of specific adhesion molecules are mechanisms that influence inflammatory cell migration and localization of cells to specific tissue sites, as blocking of adhesion molecule receptors *in vivo* can influence subsequent cellular infiltration.^{21,22,33} Inasmuch as cell trafficking and extravasation of lymphocytes to the lung may be controlled by the differential expression of these endothelial and/or leukocyte adhesion molecules, dysregulation of these processes can lead to immunopathological disease. Herein, we show that pulmonary infiltration by CD8⁺ T lymphocytes was highly associative with VCAM-1/VLA-4 adhesive interactions and, although HIV infection and AIDS were requisite for the condition of LIP, co-infection with Epstein-Barr virus (EBV) significantly compounded the severity and character of disease.

Materials and Methods

Patients and Tissue Samples

Lung was obtained at autopsy or by open-chest biopsy from 11 prepubescent children (median age, 3.6 years) with perinatally acquired HIV-1 infection and classified as P2-C (symptomatic) according to the CDC Control and Prevention classification system. All subjects had radiological evidence of pulmonary interstitial reticulonodular infiltration (widespread subsegmental consolidations) in the absence of pulmonary opportunistic infections, including *Pneumocystis carinii*, or neoplasia by standard histopathological, cytological, and/or microbiological culture and staining techniques. Exceptions were patients with human herpesvirus infections, where virus was detected prospectively in lung by *in situ* hybridization. Of these 11 cases, 6 had histological evidence of moderate to severe LIP, and of these 6, 3 showed vascular lesions manifest by thickening of the arterial intima (Table 1). The microscopic features of the vascular lesions and their association with HIV and other infectious agents were not known before the initiation of the study. Samples from the remaining five children were diagnosed with mild non-specific interstitial pneumonitis, and none had pulmonary vascular lesions. In addition, postmortem lung was obtained from children without HIV infection or pneumonia ($n = 4$) and from children with AIDS but without LIP or pulmonary arteriopathy ($n = 2$). For *in situ* hybridization and most immunohistochemical analyses, representative sections of lung were fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned to a thickness of 5 μ m, and mounted on silane-treated (triethoxyaminopropyl-silane, American HistoLabs, Gaithersburg, MD) glass microscope slides. Lung tissue was also snap-frozen in 2-methylbutane and embedded in OCT compound (Miles, Elkhart, IN) for use with antibodies that precluded aldehyde fixation. Serial sections were cut on

a cryostat microtome at a thickness of 7 μm for immunohistochemical analyses and 10 μm for tissue adhesion assays and allowed to air dry at room temperature for 2 hours.

Histochemistry

Tissues were evaluated by hematoxylin and eosin (H&E) stains for patterns of leukocyte infiltration consistent with LIP. In addition, special stains, including Masson's trichrome for collagen, Weigert's resorcin fuchsin for elastic fibers, Von Kossa's for mineral, Congo red for amyloid, and phosphotungstic acid hematoxylin for fibrin, were used to characterize the composition of vascular lesions in paraffin-embedded tissues. When available, frozen sections were also evaluated for lipid by staining with oil red o.

Immunohistochemical procedures were used to 1) identify cell types within inflammatory and/or arteriosclerotic lesions, 2) identify cells expressing viral antigens, and 3) determine the level of expression of endothelial and leukocyte adhesion molecules in histological sections of lung from individuals identified in Table 1. Immunophenotyping was performed following standard procedure,^{34,35} which included an antigen retrieval step when necessary (steaming in 1 mmol/L citrate buffer, pH 6.0, for 10 to 20 minutes) and used monoclonal antibodies (MAbs; 10 to 20 $\mu\text{g/ml}$; MAb clones and sources are identified in figure legends) to viral and cellular antigens. Briefly, primary MAbs were applied overnight at 4°C followed by detection with isotype-specific secondary antibody and an ABC peroxidase technique using 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, CA) as the chromogen. To control for nonspecific binding, isotype-matched antibodies for irrelevant antigens were used in substitution of the primary antibody. Stained tissues were evaluated by incident light microscopy.

Tissue Adhesion Assay

Leukocyte adhesion to vascular endothelium was assessed by a modified tissue adhesion assay first described by Stamper and Woodruff³⁶ and used more recently by Sasseville et al.²¹ Briefly, frozen sections were pretreated with RPMI 1640 containing 10% fetal bovine serum for 10 minutes at 4°C. Next, 10^6 CD20⁺ B cells (human B cell line Ramos) or 10^6 CD8⁺ T cells (primary clones LN2A3, LN33D7-797, and LN9E12, as described previously³⁷) were diluted in 100 μl of medium and applied to each tissue section, and the section and cells were gently shaken for 30 minutes at 4°C. After mitogen stimulation for 48 hours (5 $\mu\text{g/ml}$ pokeweed mitogen for B cells and 5 $\mu\text{g/ml}$ phytohemagglutinin for T cells; Sigma Chemical Co., St. Louis, MO), the clones were shown to express both VLA-4 $\alpha 4$ and $\beta 1$ subunits when assessed by immunocytochemistry (Figure 1P, inset) and by flow cytometry. In addition, unstimulated BCBL-2 cells, a B cell lymphoma-derived cell line,^{38,39} did not express VLA-4 $_{\alpha 4\beta 1}$ and were therefore used to control for nonspecific adhesion. The sections were then gently rinsed in

cold 0.15 mol/l PBS, fixed in 1% glutaraldehyde in PBS for 15 minutes at 4°C, rinsed in PBS/0.2% gelatin (pH 7.2), and subsequently stained with 0.5% toluidine blue/30% ethanol for 15 to 20 seconds. After rinsing in 100% ethanol, the stained sections were mounted and examined microscopically. Experiments were performed a minimum of five times.

VCAM-1 was the only endothelial adhesion molecule routinely expressed in LIP and was not up-regulated in tissues without lymphocytic lesions. Also, CD8⁺ T cells were the primary infiltrate in LIP. Thus, our investigation focused primarily on the neutralizing or blocking effects of antibodies to VCAM-1 on tissues and antibodies to VLA-4 $_{\alpha 4\beta 1}$ on CD8⁺ T cell lines. Still, a similar approach was used to assess the potential role of other adhesion pathways in LIP, including ICAM-1/LFA-1 $_{\alpha 1\beta 2}$ and E-selectin. Briefly, before the tissue-cell incubations, tissue sections, cells, or both were incubated for 30 minutes at 4°C with saturating concentrations (20 to 40 $\mu\text{g/ml}$) of MAbs to VCAM-1 (clone 51-10C9, PharMingen, San Diego, CA) or the $\alpha 4\beta 1$ subunits of VLA-4 (clones HP2/1 and Lia1/2, respectively, Coulter-Immunotech, Westbrook, ME). Monoclonal antibodies to CD31 (pan-endothelial (clone JC/70A), DAKO, Carpinteria, CA), and CD45 (leukocyte common antigen, (clone HI30), PharMingen, San Diego, CA) were used as controls on tissues and cell lines, respectively.

In Situ Hybridization

HIV-1 RNA probes were synthesized with ¹²⁵I-labeled CTP (Amersham Corp., Arlington heights, IL). Five sense and five antisense probes, in all representing 90% of the HIV-1 genome, were synthesized using pGem 3 subcloned restriction fragments of the clone HXB2, as described previously.⁴⁰ The probes were then applied to tissues as a cocktail. For EBV, 30-bp antisense oligonucleotide probes, one complementary to the EBER-1 gene transcript and another to the BHLF-1 gene transcript were prepared by labeling the 3' end with digoxigenin-11-dUTP (DIG) using a DIG-tailing reaction (Genius 6 kit, Boehringer Mannheim, Indianapolis, IN). The probes were applied to tissues as described previously.⁴¹ The EBER-1 gene is actively transcribed in latently infected cells, and the BHLF-1 gene is expressed early in the EBV life cycle and therefore identifies EBV-infected cells in the replicative phase of the virus life cycle. In addition, DIG-labeled oligonucleotide probes specific for the cytomegalovirus (CMV) early gene transcript were used following manufacturer recommendations (Novocastra, Newcastle, UK) and as reported previously.⁴² Expression of the CMV early gene transcript precedes DNA replication, and therefore, the probe allows for the earliest detection of permissive infection. Last, DIG-labeled RNA probes complementary to the Kaposi sarcoma herpesvirus (KSHV) T0.7 transcript (155 bp) were constructed as described previously.^{38,39} The T0.7 gene product is transcribed at a high molar ratio during viral latency. Positive and negative controls consisted of cytocentrifuge preparations of primary peripheral blood mononuclear cells (PBMCs)

and/or lung tissues known to be positive or negative for the respective virus by DNA extraction followed by solution-based polymerase chain reaction (PCR) and liquid hybridization. Hybridization controls consisted of noncomplementary (sense-strand) ^{125}I -labeled RNA probes for HIV and noncomplementary DIG-labeled probes for KSHV, EBV, and CMV of similar length and G/C content as the antisense probe. After development, the slides were examined by incident light and/or dark-field microscopy.

Semiquantitative Analysis

LIP was defined as infiltration of the pulmonary interstitium with mostly CD8^+ T lymphocytes and in some individuals by expansion of the alveolar septae with dense aggregates of polymorphic and polyclonal (CD19^+ and CD20^+) B lymphocytes. The severity of LIP was scored using a system we described previously^{35,43}: +, multifocal interstitial leukocytes; ++, multifocal and intermittent confluent areas of leukocyte infiltration; and +++, confluent areas of alveolar septal thickening with leukocytes and multifocal areas of lymphocyte aggregation (Table 1).

The degree of expression of endothelial VCAM-1, ICAM-1, and E-selectin was assessed semiquantitatively using a method modified from that previously described.⁴⁴ Briefly, both numbers of immunoreactive pulmonary vessels in a 0.5-cm^2 sample and intensity of staining were used as criteria. For relative numbers of immunoreactive vessels, assigned scores included the following: 0, no vessels; 1, 1 to 4 vessels; 2, 5 to 10 vessels; 3, 11 to 15 vessels; 4, 16 to 20 vessels; and 5, >20 vessels. The scores for staining intensity were as follows: 0, no reactivity; 1, faint; 2, moderate (± 4 vessels being intense); 3, intense staining of >4 pulmonary vessels. The sum of these two values for numbers and intensity of immunoreactive vessels were then used as the final score, which had a theoretical range from 0 to 8. Each sample was evaluated independently and in a blind fashion by two reviewers (S.J. Brodie and K. Diem). The severity of LIP, extent of macrophage viral burden, and levels of vessel immunoreactivity were compared using a

Spearman nonparametric correlation analysis where r values >0.7 and $P > 0.05$ were considered significant.

Cell adhesion to tissue sections was assessed by computerized image analysis. Images from 10 representative $10\times$ microscopic fields were transmitted to a computer equipped with a digital imaging board and software for determination of point count size from which a percentage of specifically stained cells per linear unit (0.2 mm) of endothelium was then determined. We chose 0.2 mm as the unit of measurement. This allowed for the inclusion of most intralesional pulmonary venules. Next, a binding coefficient was determined by the quotient of bound cells/vascular dimension unit.²¹ Cells that bound to venular endothelium were defined as those adhering exclusively to the luminal (apical) endothelial surface and were differentiated from resident or inflammatory leukocytes by concurrent assessment of untreated consecutively sectioned tissues. At least 10 vessels ≥ 0.2 mm in circumference from each tissue section were used to obtain a mean binding coefficient (MBC). Data were analyzed by one-way analysis of variance (ANOVA) to test whether the mean differed among groups of individuals categorized by HIV status and histopathology (Table 2). A Bonferroni multiple comparison test was then used as a post-test to compare pairs of group means where $P > 0.05$ was considered significant. Inhibition of cell adhesion was measured as the percentage of MBC obtained using blocking or control antibodies relative to the MBC obtained from a serial tissue section in the same assay without antibody treatment. To determine whether cell binding occurred on endothelium expressing VCAM-1, ICAM-1, or E-selectin, after performing the adhesion assay, tissue sections were post-fixed in 0.5% glutaraldehyde. An immunoperoxidase technique was then performed on the same tissue section using antibodies to VCAM-1 (clone 51-10C9, PharMingen, San Diego, CA), ICAM-1 (clone HA58 (CD54), PharMingen) or E-selectin (clone 68-5H11 (CD62E), PharMingen), respectively. Immediately after chromogen development, the tissue sections were post-fixed in 2% paraformaldehyde/0.5% PBS for 10 minutes at 25°C , washed in PBS/0.2% gelatin,

Figure 1. Infiltrative and proliferative lesions associated with pediatric lymphocytic interstitial pneumonia. Immunoperoxidase (IP) procedures used 3,3'-diaminobenzidine as the chromogen, and *in situ* hybridization (ISH) procedures used anti-digoxigenin-alkaline phosphatase (α DIG-AP) or ^{125}I -labeled CTP as an indicator. **A:** Widespread thickening of the pulmonary interstitium with mononuclear leukocytes and a single peribronchiole and perivascular aggregate of lymphocytes. Note that the expansion of lymphocytes is associated with constriction of a medium-sized artery (**arrow**) H&E, magnification, $\times 100$. **B:** Perifollicular aggregation of CD8^+ T lymphocytes. IP, CD8 (clone C8/144B, DAKO); magnification, $\times 250$. **C:** Lymphoid aggregate showing a predominance of immature B cells within the germinal center. IP, CD19 (clone 4G7/2E, Novocastra, Newcastle, UK); magnification, $\times 100$. **D to F:** Most germinal center lymphoblasts were mitotically active (**D**); IP, PCNA (clone PC10, DAKO); magnification, $\times 250$) and surrounded well developed networks of FDC (**E** and **F**, **arrows**); IP, CD21 (clone 1F8, DAKO); magnification, $\times 100$. **F:** Alveolar and interstitial macrophages were found in high numbers on the periphery of lymphoid follicles. IP, CD68 (KP1, DAKO); magnification, $\times 100$. **G:** A high percentage of cells ($>20\%$) within the follicular germinal center of lymphoid aggregates contained high copy numbers of EBV EBV-1 RNA. ISH α DIG-AP for EBV-1; magnification, $\times 100$. Most of these EBV RNA-positive cells were of B cell lineage (**G**, **inset**; combined ISH α DIG-AP for EBV-1 and IP for CD20; magnification, $\times 400$), and many expressed EBV latent membrane proteins (**H**, IP for EBV LMP (clone CS1-4, DAKO); magnification, $\times 200$). **I:** EBV also co-localized to follicular dendritic cells (**arrows**; combined ISH α DIG-AP for EBV RNA and IP for CD21 FDC; magnification, $\times 400$), but not to interstitial T lymphocytes **J**; combined ISH α DIG-AP for EBV RNA and IP for CD3; magnification, $\times 400$). **K:** HIV mRNA was observed within follicular germinal centers (^{125}I ISH for HIV-1 mRNA, dark-field; magnification, $\times 40$) in association with FDC (combined ^{125}I ISH for HIV-1 mRNA and IP for CD21 FDC; **inset**, $\times 1000$); however, cells that expressed viral antigens were found mostly on the periphery of lymphoid aggregates in areas normally occupied by macrophages (**L**, **arrows**; IP for HIV gag p24 (clone Kal-1, DAKO; magnification, $\times 200$). **M** and **N:** Serial tissue sections hybridized with noncomplementary HIV RNA probes transcribed in sense-strand orientation did not form hybrids (**M**, ^{125}I ISH for HIV-1 RNA, dark-field; magnification, $\times 40$; **N**, ^{125}I ISH for HIV-1 RNA; magnification, $\times 500$). **O:** VCAM-1 expression was heightened in vessels with perivascular inflammatory foci. IP, CD106 (clone 51-10C9, PharMingen); magnification, $\times 100$. **P:** Tissue adhesion assay demonstrating that CD8^+ T cell clones that co-express the VLA-4 ligand (**inset**, IP for VLA-4 $_{\alpha 4/\beta 1}$; magnification, $\times 200$) selectively adhere to venular endothelium in the lung from a child with severe LIP (IP for VCAM-1; magnification, $\times 400$). **Q:** Pretreatment of sequential tissue sections (shown in **P**) with blocking antibodies to VCAM-1 and incubation of CD8^+ T cell clones with blocking antibodies to VLA-4 $_{\alpha 4/\beta 1}$ inhibited $>80\%$ of this adhesion. After the adhesion assay, the tissues were stained with antibody to VCAM-1 to demonstrate presence of VCAM-1 expression. IP for VCAM-1; magnification, $\times 400$.

stained for 15 to 20 seconds in toluidine blue/30% ethanol, rinsed two times in 100% ethanol, and mounted.

Results

LIP Is an Infiltrative and Proliferative Disease

The six subjects with LIP (Table 1) all showed widespread hyperplasia of bronchus-associated lymphoid tis-

sue (BALT) and lymphocyte infiltration of alveolar septae (Figures 1A and 3A). BALT, defined as resident organized lymphoid tissue of the lung, was observed only in a small percentage (one of six) of children without evident pulmonary infection. This finding is consistent with other studies where BALT was present in human fetal and infant lung only when there was evidence of antigen stimulation.⁴⁵ Lymphocytes of CD8⁺ T cell lineage represented the predominant intraseptal infiltrate (Figure

Table 2. Mean Binding Coefficient (MBC) for CD8⁺ T Cell Clones and Ramos Cells (B Cells) to Pulmonary Vessels in Children with AIDS-Associated LIP, Nonspecific Pneumonitis, and Histologically Normal Lung

Group	Cell lines	
	CD8 ⁺ T cells	CD20 ⁺ B cells (Ramos)
HIV ⁺ , LIP (n = 6)	22.2 ± 1.9	12.8 ± 1.2
HIV ⁺ , nonspecific pneumonitis (n = 5)	2.3 ± 0.7	1.0 ± 0.3
HIV ⁺ , normal lung (n = 2)	0.7 ± 0.2	0.0
HIV ⁻ , normal lung (n = 4)	0.8 ± 0.1	0.0

Tissue adhesion assays used to determine MBCs were performed a minimum of five times. Values represent average MBC ± SEM per linear unit (0.2 mm) of venular endothelium. Data were analyzed by one-way ANOVA to test whether the mean differed among groups. A Bonferroni multiple comparison test was then used as a post-test to compare pairs of group means. Differences were present in HIV⁺ children with LIP when comparing the MBC of CD8⁺ T cell clones and Ramos B cells with other groups ($P < 0.001$). All other intra-group comparisons were insignificant ($P > 0.05$).

1B). Three of these six individuals also showed dense interstitial perivascular aggregates of polymorphic B lymphocytes (Figure 1C), most in replicative stages of the cell cycle (Figure 1D) and all surrounding well developed networks of follicular dendritic cells (FDCs; Figure 1, E and F (double arrow)). These aggregates of B cells were predominantly immunoblasts and showed normal to slightly elevated levels of mitotic activity (two to four mitotic figures per 100 cells). In addition, 20% to 30% of these B cells contained EBV EBEB-1 gene transcripts (Figure 1G) and/or EBV latent membrane proteins (Figure 1H). Herpesviral gene products localized to B cells (Figure 1G, inset) and FDCs (Figure 1I) and not to other cell types, including T cells (Figure 1J). HIV mRNA (Figure 1K) and protein antigens also localized within follicular germinal centers (Figure 1K) in association with FDCs. Loosely scattered cells within the thickened pulmonary interstitium and pockets of cells on the periphery of lymphocyte aggregates contained high copy numbers of HIV *gag* proteins (Figure 1L), areas shown previously to harbor alveolar and interstitial macrophages (Figure 1F). By combining immunohistochemistry for cell-surface antigens with *in situ* hybridization for HIV mRNA, cells that contained high viral copy numbers were mostly FDCs in germinal centers (Figure 1K, inset) and macrophages within interseptal spaces.

Arterial Intimal Proliferation

Pulmonary arteritis and/or thickening of the arterial intima was strongly correlative with severe LIP (Table 1). Lesions were present in mostly medium-sized arteries and included components of inflammation, intimal fibrosis, fragmentation of elastic tissue, and/or fibrosis or calcification of the media. In the most severely affected vessels, the endothelium was focally detached from the underlying intima and the cells were slightly rounded. Vessels demonstrating intimal thickening showed vari-

able degrees of luminal narrowing (Figure 2A) with fragmentation and/or duplication of the internal elastic lamina (Figure 2B). The thickened intima consisted of mostly collagen (Figure 2C), smooth muscle (Figure 2D), and occasional macrophages, some harboring the HIV genome (Figure 2E). In addition, one patient (case 6) showed extensive mineral deposits that localized mostly within the adventitia of medium-sized arteries (Figure 2F). Other special stains revealed the absence of amyloid, lipid, and fibrin within the vascular wall. Platelet and fibrin thrombi could be visualized within intimal plaques and within the lumen of smaller arteries. Fibrinoid necrosis, a feature of pulmonary infarction, was not present in any of the samples examined, nor did any of the subjects show lesions suggestive of pulmonary hypertension and/or congestive heart failure, such as widespread pulmonary edema. Tissues from children diagnosed with nonspecific interstitial pneumonitis, a common sequelae to HIV infection in adults,⁴⁶ and children with AIDS but without lung involvement had no evidence of vascular disease. Collectively, these findings suggest an association between pediatric LIP and arteriopathy in children with HIV-induced immune suppression.

VCAM-1/VLA-4 Adhesive Interactions

Three common and distinct pathways mediating mononuclear cell adhesion to pulmonary endothelium were investigated. VCAM-1 expression was significantly heightened in vascular endothelium in children with LIP and was most pronounced in veins with perivascular inflammatory foci (Figures 1O and 3A) and included all tissues with arteriosclerotic lesions (Table 1). Interestingly, arterial endothelium from vessels with thickened intima expressed low or undetectable levels of VCAM-1, and there was no evidence of cells with HIV *gag* mRNA or p24 antigen expression within the vascular wall. When ranking the intensity of VCAM-1 expression with a range in score from 0 to 8, the mean score for children with LIP was 5.5 ± 1.0 . Tissues with severe LIP, characterized by EBV-associated lymphoproliferation and arteriopathy, showed the most extensive and intense VCAM-1 staining (7.3 ± 0.7). In contrast, HIV-infected children without LIP, including those with nonspecific interstitial pneumonitis (1.6 ± 0.5) and those without pulmonary lesions (0.9 ± 0.4) showed low levels of VCAM-1 immunoreactivity and were no different from that of HIV-seronegative children without pulmonary lesions (0.7 ± 0.2 ; $P > 0.05$, ANOVA). Thus, only in tissues with LIP was there a significant difference in VCAM-1 expression over background ($P < 0.05$, ANOVA). VLA-4, the leukocyte ligand for VCAM-1, was expressed at high density on perivascular lymphocytes (Figure 3B), most of which also bore the CD8 surface receptor.

In contrast with VCAM-1, widespread and uniform expression of endothelial ICAM-1 was observed in sections of lung from all 11 test subjects, as well as samples from the 2 HIV-positive and 4 HIV-negative patients without pulmonary lesions (not shown). Variations in staining intensity were not appreciable. Interestingly, ICAM-1 was

Figure 2. Histochemical and immunochemical characterization of pulmonary arterial lesions. **A:** Marked intimal thickening (arrows) with luminal narrowing. H&E; magnification, $\times 100$. **B:** Disruption of the internal elastic laminae (arrows) due to expansion of the tunica intima (*I*). Weigert's resorcin fuchsin; magnification, $\times 200$ (inset). **C** and **D:** Advanced narrowing of the vascular lumen (arrows) attributable to intimal fibrosis (**C**; Masson's trichrome; magnification, $\times 100$) and smooth muscle cell proliferation (**D**; immunoperoxidase for α -actin (clone 1A4, DAKO); magnification, $\times 100$). **E:** Localization of cells harboring high copy number HIV gag mRNA (arrows) within the wall (*w*) of a medium-sized artery (*a*) and bronchiole (*b*). ^{125}I ISH; magnification, $\times 100$. **F:** Mineral deposition surrounding the tunica adventitia (*a*) of a medium-sized artery. Von Kossa's; magnification, $\times 100$.

also expressed on smooth muscle cells within the intima of affected vessels. In turn, E-selectin was only weakly and sporadically expressed on endothelial surfaces (not shown) and mostly from patients with nonspecific pneumonitis, many of which also showed chronic-active inflammation.

Using a tissue adhesion assay, we show that CD8^+ T cell clones selectively adhere to endothelium in lung from children with LIP (Figure 1P). The MBC for CD8^+ T cells

(VLA-4 $_{\alpha 4\beta 1}$ positive) was significantly greater when comparing individuals with LIP (22.2 ± 1.9) with those with nonspecific pneumonitis (2.3 ± 0.7 ; $P = 0.02$, ANOVA) or to individuals without pulmonary lesions (0.7 ± 0.2 ; $P < 0.001$, ANOVA; Table 2). There was no difference when comparing individuals with moderate (20.5 ± 2.6 ; $n = 3$) and severe (23.8 ± 3.1 ; $n = 3$) LIP ($P = 0.4$, ANOVA). Similar observations were made between the same groups when comparing tissues for Ramos cell (B cell)

Figure 3. Mechanisms of lymphocyte infiltration and localization of virus in pediatric lymphocytic interstitial pneumonia. **A:** Longitudinal section of a vein showing VCAM-1 expression and associated mononuclear cell infiltrates. IP for VCAM-1 (clone 51-10C9, PharMingen); magnification, $\times 100$. The **inset** shows a transverse section of a venule and demonstrates the uniform expression of endothelial VCAM-1. IP for VCAM-1; magnification, $\times 400$. **B:** Perivascular aggregation of CD8⁺ T lymphocytes in areas of interstitial collapse. IP for CD8 (clone C8/144B, DAKO); magnification, $\times 400$. **C:** Localization of cells harboring HIV mRNA (**arrows**) within a thin-walled vein. ¹²⁵I ISH for HIV-1 mRNA; magnification, $\times 100$. **D:** Expression of CMV early gene RNA in cytomegaloid cells (**arrows**) adjacent to a vein and in a region of interstitial collapse and lymphocyte infiltration. ISH α DIG-AP for EBV EBER-1 RNA; magnification, $\times 400$. **E:** Expression of KSHV latent gene transcripts in cells associated with follicular aggregation. ISH α DIG-AP for KSHV T0.7 RNA; magnification, $\times 400$.

adhesion, although the average MBCs were much lower than those of CD8⁺ T cell clones (Table 2). This decrease in binding efficiency of B cells coincided with a marked reduction in the expression of VLA-4 $_{\alpha 4\beta 1}$. In all, tissues with a high ratio of immunoreactive vessels and elevated staining intensity for VCAM-1 demonstrated the most severe lesions of LIP and conferred the greatest adhesive interactions to cells expressing the VLA-4 ligand.

Pretreatment of CD8⁺ T cell clones with blocking antibodies to VLA-4 $_{\alpha 4\beta 1}$ (clones HP2/1 and Lia1/2 (CDw49d/CD29), Coulter-Immunotechnology, Westbrook, ME) and/or pretreatment of tissues with LIP using blocking antibodies to VCAM-1 (clone 51-10C9 (CD106), PharMingen) resulted in >80% inhibition of this adhesion (Figure 1Q). For example, children with LIP had an average MBC of

22.2 ± 1.9 for CD8⁺ T cell clones per linear unit (0.2 mm) of pulmonary venular endothelium (Table 2). When these tissues and cells were pretreated with MAbs to VCAM-1 or VLA-4 $_{\alpha 4\beta 1}$, respectively, the MBC dropped to <5. In turn, Ramos cells, which also expressed VLA-4 $_{\alpha 4\beta 1}$ but at a much lower intensity than CD8⁺ T cell clones, also bound less efficiently to endothelium in serial sections of the same tissues. Unstimulated cells bound less efficiently than mitogen-stimulated cells, and unstimulated BCBL-2 cells did not adhere to vascular endothelium under any application. LFA-1 was also expressed on CD8⁺ T cell clones and Ramos cells. However, in the absence of VCAM-1 expression, neither cell type bound appreciably to endothelium that expressed ICAM-1 alone (T cells, MBC = 2.4 ± 0.8 ; Ramos cells, MBC = $0.8 \pm$

0.2; clone HA58 (CD54), PharMingen) or vessels that co-expressed ICAM-1 and E-selectin (T cells, $MBC = 1.5 \pm 0.6$; Ramos cells, $MBC = 0.7 \pm 0.2$). Similarly, pretreatment of lymphocyte cell lines with saturating concentrations of antibodies to LFA-1 $_{\alpha 1\beta 2}$ (clone HI111 (CD11a), PharMingen, and clone MHM23 (CD18), DAKO) did not significantly affect the binding of these cells when applied to tissues with vessels that expressed ICAM-1 or E-selectin alone (T cells, $MBC = 2.1 \pm 0.7$; Ramos cells, 0.8 ± 0.1 ; $P > 0.05$). Cells and tissues when incubated with antibodies to CD45 and CD31, respectively, as a control for nonspecific blocking of cell-to-tissue adhesion, did not alter the MBC in the tissue adhesion model ($P > 0.05$).

Virus Localization by In Situ Hybridization

Mononuclear cells bearing HIV transcripts were observed, although infrequently, within the wall of arteries (Figure 2E) and veins (Figure 3C) and mostly in sites of perivascular infiltration. The majority of cells with detectable HIV mRNA had morphological and phenotypic (CD68⁺) features of macrophages and localized within the interstitial and alveolar spaces (Figure 1, K and L). The number of HIV-infected intralesional monocyte/macrophages was strongly correlative with the severity of LIP ($r = -0.86$; $P = 0.01$, Spearman rank correlation coefficient, two-tailed analysis); severe lesions with both infiltrative and proliferative components (cases 1, 2, and 6) showed the greatest number of cells with detectable HIV *gag* gene expression (Table 1).

Because γ -herpesviruses have also been linked to LIP and more recently with arteriopathy,^{47,48} we examined lung for the presence of viral transcripts and protein antigens indicative of CMV, KSHV, and/or EBV infections. We also examined the effects of concurrent herpesvirus infection on adhesion molecule expression. The child with CMV pneumonitis (case 4) had no evidence of arteriopathy and showed only low levels of endothelial VCAM-1 (score 2.0). Although, VCAM-1 expression in this patient was most pronounced in vessels within areas of heavy lymphocyte infiltration, the distribution of cells expressing CMV RNA was widespread (Figure 3E) and included histologically normal areas of the lung. The role of KSHV in lesion development was also unclear as cells containing the T0.7 transcript were detected in only 1 of 11 subjects (case 1), and there were no patterns of virus localization relative to sites of lymphoproliferation, arteriosclerotic vessels, and/or vessels expressing VCAM-1. In contrast, the three patients with severe LIP (Table 1) characterized by dense intraseptal aggregates of lymphoblasts (Figure 1C), showed that as many as 20% of these cells harbored detectable levels of EBV EBER-1 RNA (Figure 1G) and/or expressed EBV latent membrane protein 1 (Figure 1H). EBV was not detected in bronchiolar or alveolar epithelium, as reported by others,⁴⁹ nor was it observed in T cells (Figure 1J).

Discussion

Determining which adhesion molecule pathway(s) are involved in a particular inflammatory disease process has important implications for understanding events responsible for leukocyte recruitment and for therapeutic intervention. We show uniform expression of VCAM-1 in tissues with LIP and not in control tissues, including those with nonspecific pneumonitis. Moreover, expression of VCAM-1 on postcapillary venules showed the highest degree of spatial localization with infiltrates. Using a tissue adhesion assay, CD8⁺ T cell attachment was blocked by incubating tissues with antibody to VCAM-1 and was maximal when blocking antibodies to both $\alpha 4$ and $\beta 1$ subunits of VLA-4 were applied concurrently. Collectively, these results suggest strongly that VCAM-1/VLA-4 adhesive interactions are important in lymphocyte trafficking in LIP. Moreover, differences in host factors in children that allow for higher viral loads^{50,51} and heightened cytokine responsiveness⁵² may ultimately influence the progression of LIP.

ICAM-1 mediates an LFA-1-dependent pathway³¹ and, like VCAM-1, may be important in lymphocyte emigration to sites of inflammation or immune reaction.^{23,53} However, unlike VCAM-1, ICAM-1 is constitutively expressed and is present on a variety of other cell types. Hence, the role of ICAM-1 in inflammation may be difficult to ascertain. Moreover, because ICAM-1 was also expressed in normal areas of diseased tissue, it was unlikely to have played a major role in leukocyte infiltration in children with LIP. E-selectin may also play a role in the initial interaction of certain subpopulations of T lymphocytes with activated endothelium.^{19,30} We show that E-selectin was expressed on venular endothelium from tissues with nonspecific pneumonitis, most often when there was a prominent neutrophil component, but was absent or only weakly expressed in tissues with LIP. Blockade of E-selectin and ICAM-1/LFA-1 $_{\alpha 1\beta 2}$ did not significantly alter lymphocyte attachment to pulmonary endothelium. Thus, ICAM-1 and E-selectin did not appear to contribute substantially to lymphocyte trafficking in LIP. Still, we cannot discount that these and other adhesion pathways, although having a minor contribution in the adhesion assay when examined individually, may work in concert *in vivo* to contribute to lymphocyte-endothelial adhesion. It is also conceivable that our assays were insensitive to reveal relatively low-affinity interactions that may occur with selectins and their ligands.

A variety of herpesviruses have also been implicated in pediatric LIP^{5,8,12,13} and expansion of BALT.⁵⁴ We show that EBV localized to B lymphoblasts in all tissues with lymphoid aggregates and also within FDCs in lesions with germinal center formation. This high incidence of EBV infection and virus replication may result from defective regulation of EBV in patients with AIDS or AIDS-related disorders.⁵⁵ Patients with LIP typically show high numbers of EBV-infected B cells in circulation as a consequence of profound defects in T cell immunity.^{55,56} Children with AIDS or AIDS-related disorders, including those with LIP, are also predisposed to EBV-associated non-Hodgkin's lymphoma.^{14,15,56} Consequently, it has been

proposed that LIP may represent an intermediate process between benign and malignant transformation.⁵⁷ Co-infection of B cells with HIV and EBV has also been described as a possible co-factor in the progression from polyclonal B cell proliferation to lymphoma as both the up-regulation of *c-myc* and activation of EBV can occur as a result of HIV infection.¹¹ Interestingly, when comparing the intensity and avidity of VCAM-1 expression and cell adhesion (MBCs) in children with LIP there was no difference when examining tissues with EBV-associated lymphoblastic lesions (++++LIP) and those without (++LIP). This further suggests that this B cell component of LIP does not occur as the result of infiltration, but results from local expansion of B cells.

The pulmonary arterio-occlusive lesions that we describe appear to be unique to children with AIDS and, interestingly, were observed only in children with concurrent EBV infection. Similar lesions have been described in macaques experimentally infected with SIV_{mac}⁵⁸ and more recently in transgenic mice carrying a replication-defective HIV-1 provirus.⁵⁹ Newly described γ -herpesviruses have also been linked to arteriopathy in SIV-infected macaques⁴⁸ and large-vessel arteritis in mice.⁴⁷ Various types of synergy have been described between retroviruses and herpesviruses.^{16–18} Of potential relevance to vascular disease, HIV-1 *tat* protein was shown to promote the growth of normal vascular cells and spindle cells derived from AIDS-associated Kaposi's sarcoma,⁶⁰ a tumor of vascular origin and common neoplasm in AIDS patients and a disease recently attributed to γ -herpesvirus infection.⁶¹

Studies of Kaposi's sarcoma cell lines indicate that KS cells release a variety of cellular growth factors.⁶² EBV-infected and/or EBV-activated lymphocytes also liberate angiogenic cytokines, including endothelial growth factor and fibroblast growth factor.^{63,64} Thus, we examined sections of lung with histological evidence of arteriopathy for the γ -herpesviruses KSHV and EBV by *in situ* hybridization. Vascular lesions were characterized by intimal thickening resulting from collagen and smooth muscle deposition and affected mostly medium-sized arteries. Only 1 of 11 subjects demonstrated cells carrying the KSHV latent genome, and unlike EBV where high levels of virus localized to B cells within lymphoid aggregates, there was no pattern to the distribution of KSHV in this patient, and viral transcripts were not detected in individuals with vasculopathy. Previous studies of ours have shown KSHV latent gene transcripts in prostate glandular epithelium of HIV-infected men but only at very low viral copy number.^{38,39} In contrast, both latent and transcriptionally active EBV infection of B cells occurred in all individuals with arterio-occlusive disease. Yet, unlike HIV, EBV was not detected within the vascular wall of affected vessels. Others have shown the absence of EBV in large-vessel disease.⁶⁵ We also examined lung for CMV, a β -herpesvirus capable of up-regulating HIV replication in macrophages^{16,66} and thought to induce smooth muscle proliferation in inflammatory aortic aneurysms.⁶⁷ Cytomegaloid cells carrying the CMV early gene were identified in the pulmonary interstitium but only in a single patient and not in association with vascular lesions or

within sites of lymphoid aggregation. Collectively, these findings suggest that EBV, and not KSHV or CMV, may play a role in pulmonary arterial disease in immunocompromised children; however, the mechanism for this potential interaction is unclear.

Herein, we show that pediatric LIP is a multifaceted disease with infiltrative and proliferative components. Infiltration of CD8⁺ T lymphocytes into the pulmonary interstitium was highly correlative with VCAM-1/VLA-4 adhesive interactions between pulmonary endothelium and blood leukocytes. In addition, individuals with arteriosclerotic lesions demonstrated multifocal aggregates of B cells that occurred in association with high levels of B cell infection with EBV. Although HIV infection and AIDS were requisite for the condition of LIP, the mechanism(s) of induction of VCAM-1 and role of EBV, as a viral co-factor or simply as an opportunistic agent, requires further investigation.

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