Evidence for the Origin of Kaposi's Sarcoma From Lymphatic Endothelium

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Previous studies utilizing enzyme histochemistry, electron microscopy, and immunohistochemistry have failed to establish the cell of origin in Kaposi's sarcoma. The authors have rigorously tested the prevailing hypothesis that the lesion defined as Kaposi's sarcoma is derived from vascular endothelial cells. They use seven markers to characterize endothelial cells: three antigens (Factor VIII-related antigen, HLA-DR/Ia, macrophage/endothelial antigens), three enzymes (5'-nucleotidase, ATPase, alkaline phosphatase), and lectin binding (*Ulex europaeus* I). They applied the markers first to normal skin and lymph node, and then to biopsy specimens from 40 patients with Kaposi's sarcoma. Normal blood vessel endothelium was positive for all seven markers, but normal lymphatic endothelium was negative for all of the markers except 5'-nucleotidase and *Ulex europaeus* lectin. The neoplastic cells in 40 cases of Kaposi's sarcoma closely resembled those of normal lymphatic endothelium but not those of blood vessel endothelium. This suggests that Kaposi's sarcoma may originate in lymphatic endothelium. (Am J Pathol 1985, 119:294–300)

KAPOSI'S SARCOMA is a distinctive tumor of the skin originally described by Moriz Kaposi in 1872.[1] The cell of origin and the nature of the disease have remained controversial for 112 years. Although Kaposi's sarcoma was previously an uncommon lesion, a recent upsurge in its incidence associated with the acquired immune deficiency syndrome (AIDS)[2] has prompted renewed interest in it and has provided an unusual opportunity to study it.

Approximately 90 years after the initial description of Kaposi's sarcoma, Tedeshi[3] and Bluefarb[4] reviewed the rather remarkable list of proposed cells of origin. The early studies, based primarily on the histologic appearance of the lesion and its clinical pattern, were followed by studies exploring its nature. These span the period of from the early 1960s to the present and have utilized enzyme histochemistry,[5–13] electron microscopy,[10,12–19] and immunohistochemistry.[19–26] Although a variety of cells were put forward as the cell of origin in these studies, the two most common suggestions were an endothelial cell or a pluripotent mesenchymal cell. The recent immunohistochemical data are based almost exclusively on the localization of Factor VIII-related antigen (FVIII RA), a protein used as a marker of endothelium.[27,28] FVIII RA was not detected in the abnormal spindle cell elements of Kaposi's sarcoma in four studies[19–22] but was detected in these elements in four other studies.[23–26] The lectin *Ulex europaeus* I, a more recently described endothelial marker,[22,32] has been applied to only a few cases of Kaposi's sarcoma. Miettinen et al demonstrated "focal positivity confined to the small vessels . . .," while Ordonez and Batsakis showed "focal staining" in the spindle cells and positive staining in "the vascular component." Thus, the cell of origin has not been unequivocally settled by these studies.

We have used two different procedures to characterize Kaposi's sarcoma by enzyme histochemistry and immunohistochemistry: 1) a modified plastic-embedding technique originally developed for the localization of enzymes[29,30] and 2) a frozen-section technique.[31] The plastic-embedding technique allows extremely precise localization of antigens and enzymes, in combination with superior morphologic preservation; whereas the frozen-section technique is considered the most sensitive antigen detection system available. We examined the tissue for the presence of three antigens (FVIII RA, HLA-DR/Ia, macrophage/endothelial antigens), three enzymes (5'-nucleotidase, ATPase, alkaline phospha-

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tase), and lectin binding sites specific for *Ulex europaeus* (UEA I). FVIII RA and HLA-DR/Ia were examined in both plastic and frozen specimens, while the macrophage/endothelial antigens were tested only in frozen sections. The enzyme and lectin studies were performed exclusively in plastic sections. We found that endothelial cells in normal tissues were nonuniform and that distinctive phenotypes could be identified for blood vessel and lymphatic endothelium. Furthermore, examination of 40 biopsy specimens of Kaposi's sarcoma revealed characteristics similar to those of normal lymphatic endothelium, but distinctly different from those of normal vascular endothelium.

Materials and Methods

Biopsy materials from 40 patients (13 skin biopsies and 27 lymph node biopsies) were studied with plastic embedding. The patients were predominantly young homosexual men with AIDS, but 2 patients were older men with the traditional form of Kaposi's sarcoma. The tissue was fixed in 4% paraformaldehyde in 0.1 M PO_4 buffer, pH 7.4, for 4–6 hours at 4 C and then embedded in plastic as previously described.[29,30] Enzyme-histochemical procedures were performed as previously described.[29,30] Immunohistochemical and lectin histochemical procedures were performed in the following sequence: Unless otherwise specified, all incubations were carried out at room temperature (RT).

1) Digestion of plastic sections with 0.25% trypsin for 45 minutes at 37 C, followed by 0.05% phenylhydrazine (pH 7.1) for 1 hour at 37 C.
2) Incubation of sections in one of the following sequences of antisera or lectin conjugate:
   a) rabbit anti-human FVIII RA antibodies directly conjugated to peroxidase (Dako) in a 1:10 dilution for 2 hours.
   b) rabbit anti-human FVIII RA antibodies (Dako and Calbiochem) in a 1:1000 dilution overnight at 4 C; biotinylated goat anti-rabbit IgG (Vector) in a 1:500 dilution for 1 hour; rabbit peroxidase–antiperoxidase (PAP) complex (Accurate) in a 1:100 dilution for 1 hour, or avidin–biotin–peroxidase complex (ABC) (Vector) in a 1:80 dilution for 1 hour.
   c) mouse anti-human HLA-DR/Ia antibodies (Coulter, I2) in a 1:500 dilution overnight at 4 C; biotinylated horse and anti-mouse IgG (Vector) in a 1:300 dilution for 1 hour; ABC complex in a 1:80 dilution for 1 hour.
   d) UEA I lectin directly conjugated to peroxidase (EY) in a 1:50 dilution for 2 hours at RT.
3) Development of peroxidase reaction product by preincubation with 0.05% 3,3-diaminobenzidine for 10 minutes, followed by incubation with 0.05% diaminobenzidine with 0.1% H_2O_2 and 0.1 M imidazole for 7 minutes; the reaction is further enhanced by incubation with 0.5% $CuSO_4$ for 5 minutes.

All antibody dilutions were made in phosphate-buffered saline (PBS) containing 3% normal goat or horse serum (depending upon which secondary antiserum was used), and sections were rinsed between incubations with the same solution.

Biopsies from 9 additional men (8 with AIDS and 1 on chronic steroid therapy) were snap-frozen, cyrostat-sectioned, and acetone-fixed as previously described.[31] Immunohistochemical procedures were performed in the following sequence:

1) Incubation of sections for 15 minutes at RT either with rabbit anti-human FVIII RA antibodies directly conjugated to peroxidase (Dako) in a 1:10 dilution or with one of the following murine monoclonal antibodies:
   a) anti-human FVIII RA (clone SVIII/44/20 supernatant provided by David Mason, Oxford), undiluted.
   b) anti-human HLA/DR/Ia (clone L203 supernatant provided by Ronald Levy, Stanford), undiluted.
   c) anti-human macrophage/endothelial (Mϕ/E) antigens (clones 63D3 and 61D3, ascites provided by Donald Capra, University of Texas at Dallas) in a 1:50 dilution; and Leu-M_3 (Becton–Dickinson) in a 1:60 dilution.
2) Incubation of sections exposed to murine monoclonal antibodies with $F(ab')_2$ fragments of purified biotinylated goat anti-mouse IgG (Tago) in a 1:50 dilution for 15 minutes followed by avidin–peroxidase complex (Vector) in a 1:80 dilution for 15 minutes at RT.
3) Development of peroxidase reaction product by incubation in 0.03%, 3,3-diaminobenzidine with 0.03% H_2O_2 for 10 minutes; enhancement of reaction product by incubation with 0.5% $CuSO_4$ for 5 minutes.

Control procedures for both methods included the substitution of irrelevant antibodies for the primary antibodies and the elimination of primary or secondary reagents. Normal vessels within the sections served as positive controls. Only the results in sections with positive control elements and clear delineation of the neoplastic element were recorded.

Results

Characterization of Normal Endothelium

Enzyme-histochemical and immunohistochemical studies revealed considerable variation in the charac-

Table 1—Characterization of Normal Human Endothelium

Endothelium	Staining With Endothelial Markers						
	FVIII RA	HLA-DR/Ia	UEA	Alkϕ	ATPase	5′-N	Mϕ/E
Lymphatic	0*	0*	+ + +	0	0	+ + +	0*
Arteriole	+ +	+ +	+ +	+ +	+ +	+	+
Capillary	+ + +	+ +	+ +	+ + +	+ +	+ +	+
Venule	+ + +	+ +	+ +	+	+ +	+	+

FVIII RA, Factor VIII-related antigen; HLA-DR/Ia, HLA-DR/Ia-like antigen; UEA, *Ulex europaeus* I lectin; Mϕ/E, macrophage/endothelial antigens; Alkϕ, alkaline phosphatase; 5′-N, 5′-nucleotidase; ATPase, adenosine triphosphatase; 0, negative; 0*, a rare weakly positive cell; +, weakly positive; ++, positive; + + +, strongly positive.

teristics of endothelium from normal lymph nodes and skin (Table 1). Although most of the small vessels on both the arterial and venous sides showed considerable similarities in staining, some differences in the degree of staining were apparent. Typically, blood vessel endothelium was positive with antibodies to FVIII RA and HLA-DR/Ia, showed strong staining with the lectin UEA I, and was positive for the enzymes alkaline phosphatase (Alkϕ), 5′-nucleotidase (5′-N), and ATPase (ATP). Staining with the macrophage/endothelial Mϕ/E) antigens was variably present in both capillaries and venules. The differences in staining between vascular and lymphatic endothelium were considerably more striking. Lymphatic endothelium lacked the enzymes Alkϕ and ATP but showed the strongest 5′-N reaction of all the vessels. There was little evidence of HLA-DR/Ia antigen, FVIII RA, or Mϕ/E antigens, but a strong reaction with UEA I was characteristic. Occasionally, a faint questionable positive reaction for FVIII RA, HLA-DR/Ia, and Mϕ/E antigens was observed in frozen sections, which suggested that the cells might produce these antigens in very small amounts. The traits provide a clear distinction between lymphatic endothelium (FVIII RA$^-$, HLA-DR/Ia$^-$, Mϕ/E$^-$, ATP$^-$, Alkϕ$^-$, 5′N$^+$) and blood vessel endothelium (FVIII RA$^+$, HLA-DR/Ia$^+$, Mϕ/E$^+$, UEA$^+$, ATP$^+$, 5′N$^+$).

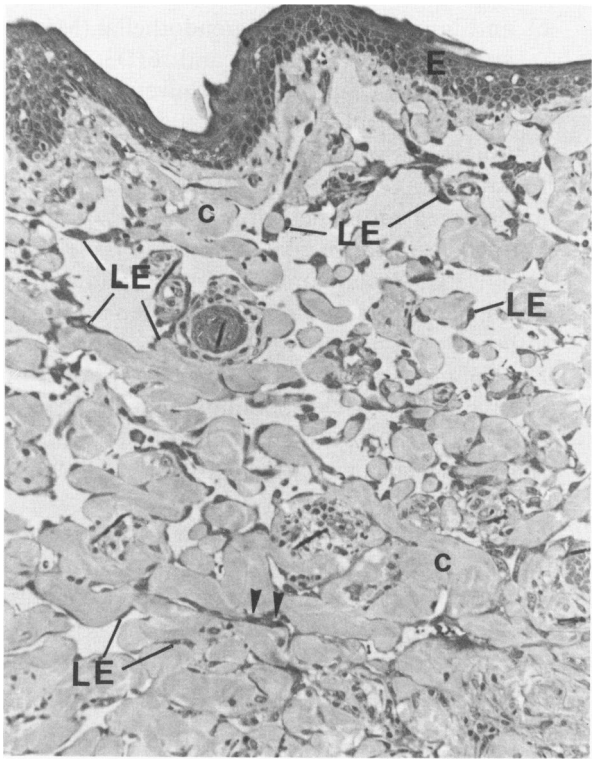

Figure 1—Early lesion of Kaposi's sarcoma in the skin. The epidermis (*E*) is uninvolved. The irregular vascular spaces, lined by plump lymphatic endothelial cells (*LE*), are well demonstrated in the upper part of the dermis. The dermal collagen (*C*) is dissected into bundles by the vascular spaces lined by the proliferating cells. The lower part of the lesion is similar, but the pattern is less obvious because the spaces are not dilated (*double arrows*). (H&E, ×125)

Characterization of Kaposi's Sarcoma

The lesions of Kaposi's sarcoma are a complex mixture of cell types. The abnormal elements range from irregular, dilated vascular channels to solid areas of spindle cells. While both ends of the spectrum were frequently present in a given lesion, some lesions were almost completely dominated by one or the other pattern.

Our observations revealed a pattern of development of the lesions. The earliest lesions in both the skin and the lymph node consist of irregular vascular spaces lined by plump endothelial cells showing the staining traits observed in normal lymphatic endothelium. These cells surround and separate preexisting collagen bundles, vessels, and nerves to form a distinctive spongy network (Figure 1). In some lesions, vascular spaces were hard to identify because they were not dilated. The early lesions generally show a modest inflammatory infiltrate. In later lesions, the structure is complicated by neovascularization, producing distinctive structures with a core composed of a morphologically and phenotypically normal capillary, venule, or arteriole, a modest amount of collagen, and moderate numbers of inflammatory cells, covered by a layer of proliferating abnormal endothelial cells (Figure 2). With further proliferation, the abnormal endothelial cells accumulate and occasionally form solid cords. The vascular cores also proliferate, apparently in response to the tumor. This

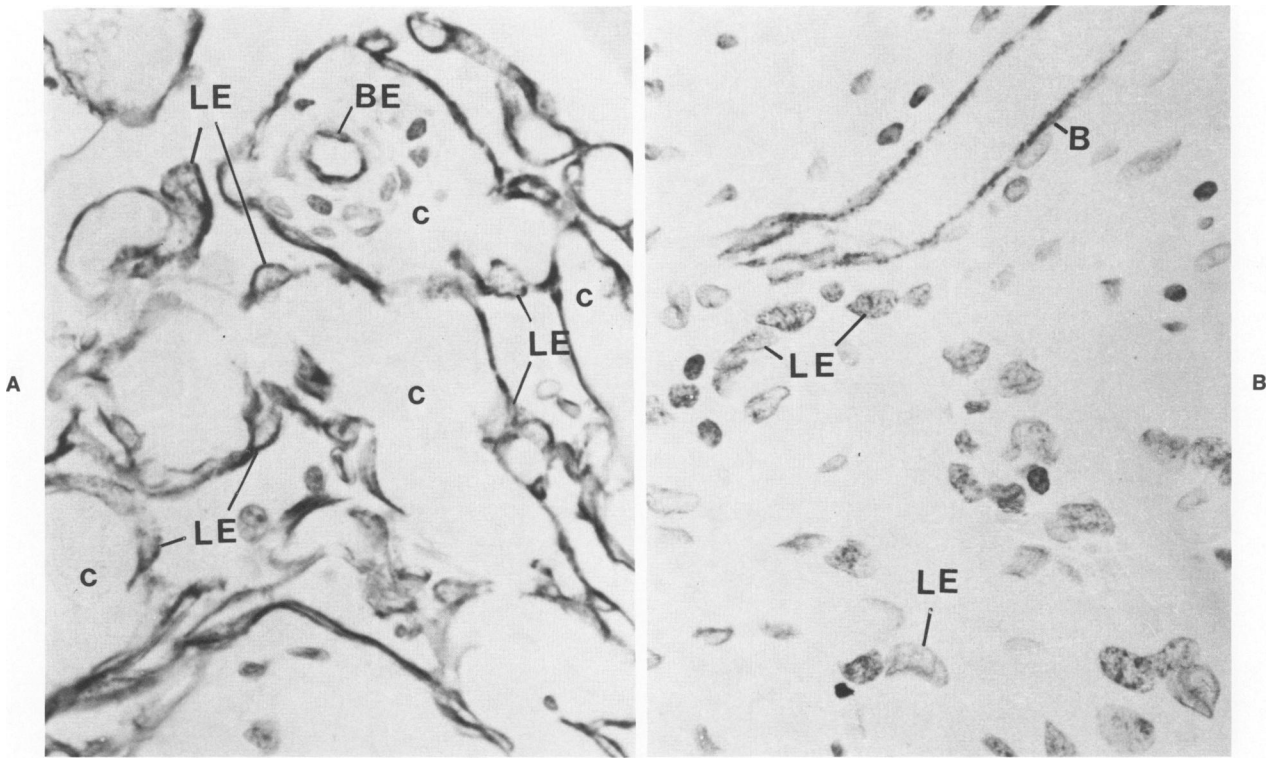

Figure 2—Early lesion of Kaposi's sarcoma labeled with *Ulex europaeus* I lectin conjugated to peroxidase (**A**) and peroxidase-conjugated antibodies to Factor VIII-related antigen (**B**). (×600) The neutral red counterstain darkens only the nuclear chromatin. In **A**, a strong black reaction for *Ulex europaeus* is apparent on abnormal lymphatic endothelial (*LE*) cells lining the irregular spaces and on normal blood vessel endothelial (*BE*) cells within the collagen (*C*) bundle. The black reaction for Factor VIII-related antigen (**B**) is confined to normal blood vessel endothelial (*BE*) cells.

proliferation produces highly vascular lesions in which the abnormal endothelial cells may be difficult to distinguish from normal endothelium without the use of markers or high-resolution plastic sections. The continued proliferation of the abnormal endothelial cells ultimately overwhelms the other elements, which results in solid sheets of spindle cells (Figure 3). Inflammatory cells, particularly macrophages, are frequently admixed.

In plastic sections, the abnormal proliferating endothelial cells showed consistent staining with UEA I and 5′-N (Figures 2 and 3), although the staining was weaker and more patchy in the solid areas of spindle cells. The other endothelial markers, FVIII RA, HLA-DR/Ia, Alkϕ, and ATP (Figures 2B and 3B), generally failed to stain the neoplastic endothelium or the spindle cells developed from it, although a single case showed weak FVIII RA staining. All the vessels in the complex network supplying the lesion showed a staining pattern consistent with normal vascular endothelium. These results are summarized in Table 2. In frozen sections the spindle cells of Kaposi's sarcoma were uniformly unreactive with antibodies against HLA-DR/Ia and Mϕ/E antigens. In seven biopsy specimens, the spindle cells were also completely unreactive with anti-FVIII RA monoclonal antibody and heteroantiserum; however, in two others there was weak reactivity with both reagents. Since occasional weakly positive staining was seen in normal lymphatic endothelium in frozen sections, this finding is not incompatible with a lymphatic origin. These observations led us to conclude that the phenotype of Kaposi's sarcoma differs significantly from that of blood vessel endothelium and more closely resembles that of lymphatic endothelium.

The problem of nonspecific background staining was addressed in both test systems. In plastic sections prepared with heteroantiserum to FVIII RA, it was apparent that the surface membranes of macrophages and some fibroblasts were binding the antibody at a dilution that caused no other background staining. Tests with sequential substitutions at various stages of the procedure suggested that this binding represented nonspecific binding of the primary antibody. We also noted that further dilutions of the primary antiserum and the addition of 3% normal serum to the diluent markedly reduced this problem. In frozen sections, normal endothelial cells usually showed no nonspecific background staining, but the spindle cells of Kaposi's sarcoma exhibited moderate or strong background

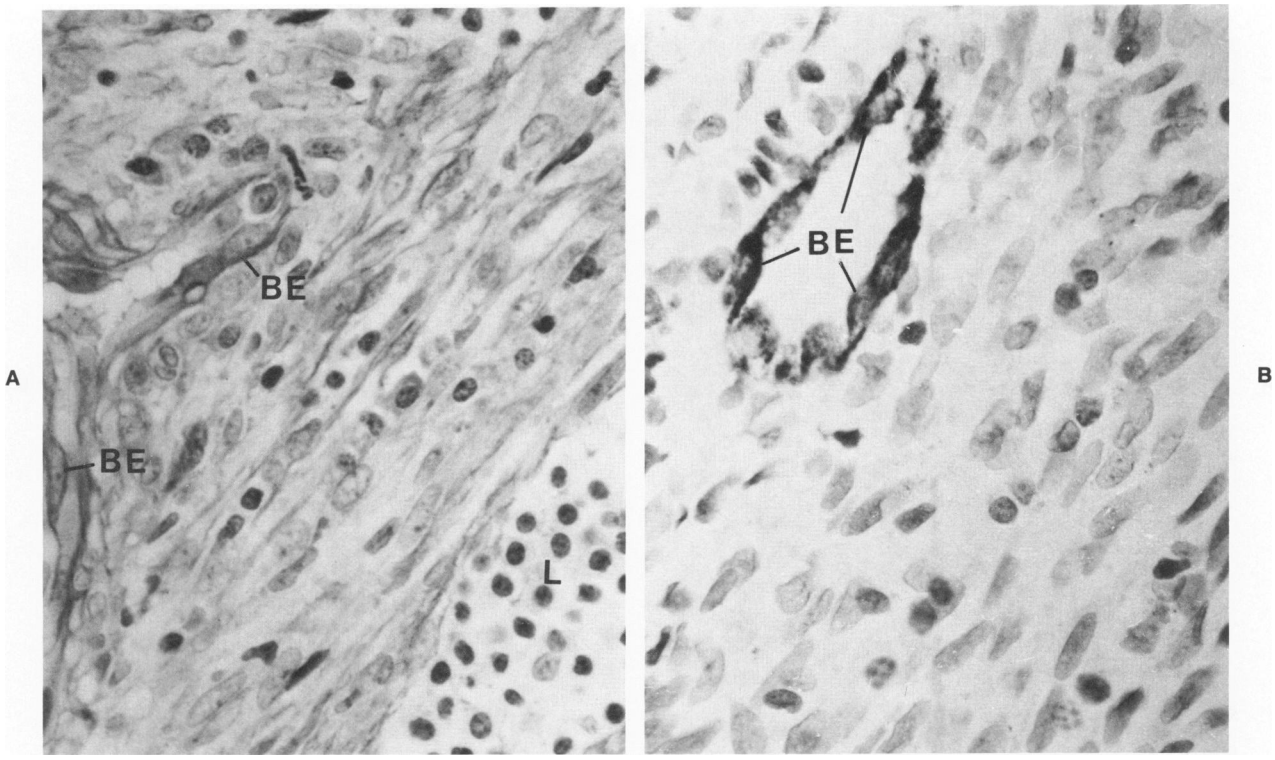

Figure 3—Late-stage spindle cell area of Kaposi's sarcoma tested for 5′-nucleotidase (**A**) and peroxidase-conjugated antibodies to Factor VIII-related antigen (**B**). (×600) The neutral red counterstain darkens only the nuclear chromatin. In **A**, both normal blood vessel endothelial cells (*BE*) and spindle cells show dark reaction product on their membranes, while an area of residual lymphoid (*L*) cells shows no staining. In **B**, only the normal blood vessel endothelium (*BE*) is stained. The spindle cells are completely negative.

staining for five of nine biopsy specimens. Control procedures, including the sequential substitution of reagents at various stages, indicated two sources of background staining: 1) nonspecific binding of primary reagents and 2) endogenous peroxidase activity in extravasated erythrocytes.

Discussion

This study demonstrates that the phenotype of the abnormal proliferating cell in Kaposi's sarcoma differs significantly from that of nonneoplastic blood vessel endothelium. The phenotype demonstrated (FVIII RA^-, HLA-DR/Ia^-, $M\phi/E^-$, UEA^+, ATP^-, $Alk\phi^-$, $5'\text{-}N^+$) closely resembles the phenotype of normal lymphatic endothelium. These data suggest that Kaposi's sarcoma is a proliferative disorder of lymphatic endothelial cells. Further support for this contention comes from the elegant electron-microscopic studies of McNutt et al,[18] who found that early Kaposi's sarcoma lesions were remarkably similar to dermal lymphatics. Dorfman[7] provided the first experimental evidence that the lym-

Table 2—Characterization of Kaposi's Sarcoma

	Staining with endothelial markers*						
Cell type	FVIII RA	HLA-DR/Ia	UEA	Alkϕ	ATPase	5′-N	Mϕ/E
Plastic sections							
Abnormal endothelial cells of Kaposi's sarcoma	0/34	0/35	30/36	0/40	0/38	27/31	ND
Spindle cells of Kaposi's sarcoma	1/22	0/20	15/22	0/25	0/23	5/16	ND
Frozen sections							
Spindle cells of Kaposi's sarcoma	2/9	0/9	ND	ND	ND	ND	0/9

FVIII RA, Factor VIII-related antigen; HLA-DR/Ia, HLA-DR/Ia-like antigen; UEA, *Ulex europaeus* I lectin; Alkϕ, alkaline phosphatase; ATPase, Adenosine triphosphatase; 5′-N, 5′-nucleotidase; ND, not done.

* Data are given as number of cases positive per number of cases tested with adequate survival of marker. Only solid clusters of abnormal spindle cells are tabulated so that confusion with reactive stromal elements is avoided.

phatic endothelial cell was the cell of origin, although this hypothesis had been suggested earlier by several other investigators.

The conflicting results previously obtained with FVIII RA warrant comment. Our results were almost completely negative, although an occasional case showed weak FVIII staining compatible with rare weakly positive cells observed in normal lymphatics. As noted earlier, several investigators,[19-22] using paraffin sections, found no staining for FVIII RA in the abnormal spindle cells of Kaposi's sarcoma. Others, using paraffin and frozen sections,[23-26] detected staining in the abnormal cells. It has been suggested that some variations might be due to differences in the source of the antibody, but our review of the studies revealed no consistent pattern related to antibody source, and we saw no significant difference in the reactions using four different antibodies. It should also be noted that spurious staining in paraffin sections is a well-recognized phenomenon. False-negative as well as false-positive results have been described,[33] and they are not necessarily detectable by the substitution of normal antiserum for primary antisera, because the primary antisera may be cross-reactive with unintended antigens. Specifically regarding FVIII RA, a recent report indicates that a variety of tumors will yield false-positive results when studied in paraffin sections.[34] The lesions of Kaposi's sarcoma are extremely complex and well vascularized, further complicating the interpretation of data. The plastic sections we used completely eliminate any localization problems. Finally, although it is possible that our plastic technique might be insensitive to extremely small amounts of FVIII RA detectable by other techniques, it is unlikely that significant amounts of FVIII RA were undetectable in frozen sections.

We conclude that the irregular vascular spaces in the earliest recognizable lesions of Kaposi's sarcoma are formed by proliferating cells most closely resembling the cells of normal lymphatic endothelium. The morphologic appearance of these lesions is complicated by an inflammatory infiltrate and neovascularization by phenotypically normal blood vessels. Whether these two components represent a reaction to the proliferating lymphatic endothelium or a more fundamental pathologic event remains unclear. With continued proliferation some portions of the lymphatic endothelial elements lose the ability to form channels and show a considerable loss of UEA I binding and 5'-N. The observation of clear transitions between these two states suggests that the spindle cell element is derived from the proliferating lymphatic endothelium. These studies suggest that the cell of origin of Kaposi's sarcoma is the lymphatic endothelial cell.

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