ANTIBODIES TO BUTYRATE-INDUCIBLE ANTIGENS OF KAPOSI'S SARCOMA–ASSOCIATED HERPESVIRUS IN PATIENTS WITH HIV-1 INFECTION

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Abstract *Background.* The recent identification in patients with Kaposi's sarcoma of DNA sequences with homology to gammaherpesviruses has led to the hypothesis that a newly identified virus, Kaposi's sarcoma–associated herpeslike virus (KSHV), has a role in the pathogenesis of Kaposi's sarcoma. We developed serologic markers for KSHV infection.

Methods. KSHV antigens were prepared from a cell line (BC-1) that contains the genomes of both KSHV and the Epstein–Barr virus (EBV). We used immunoblot and immunofluorescence assays to examine serum samples from 102 patients with human immunodeficiency virus type 1 (HIV-1) infection for antibodies to KSHV-associated proteins and to distinguish these antibodies from antibodies to EBV antigens. A positive serologic response was defined by the recognition of an antigenic polypeptide, p40, in *n*-butyrate–treated BC-1 cells and by the absence of p40 recognition in untreated BC-1 cells or EBV-infected, KSHV-negative cells. The detection by the immunofluorescence assay of 10 to 20 times more antigen-positive cells in *n*-butyrate–treated BC-1 cells than in untreated cells was considered a positive response.

Results. Antibodies to the p40 antigen expressed by chemically treated BC-1 cells were identified in 32 of 48 HIV-1–infected patients with Kaposi's sarcoma (67 percent), as compared with only 7 of 54 HIV-1–infected patients without Kaposi's sarcoma (13 percent). These results were confirmed by an immunofluorescence assay. The positive predictive value of the serologic tests for Kaposi's sarcoma was 82 percent, and the negative predictive value 75 percent.

Conclusions. The presence of antibodies to a KSHV antigenic peptide correlates with the presence of Kaposi's sarcoma in a high-risk population and provides further evidence of an etiologic role for KSHV. (N Engl J Med 1996;334:1292-7.)

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K APOSI'S sarcoma, a multifocal vascular tumor, was first described by the Hungarian-born dermatologist Moritz Kaposi in 1872.[1] The classic form predominantly affects men of Eastern European and Mediterranean heritage.[2] Kaposi's sarcoma also occurs in immunocompromised patients, particularly homosexual and bisexual men with human immunodeficiency virus type 1 (HIV-1) infection and allograft recipients receiving immunosuppressive therapy.[3-7] An endemic form that does not involve HIV is found in central Africa.[8]

The possibility that an infectious agent is involved in the pathogenesis of Kaposi's sarcoma is suggested by the clustering of the disease in well-defined populations and the relation of the disease to immunosuppression. Several agents have been investigated as etiologic factors, including cytomegalovirus, the human papillomavirus, and *Mycoplasma penetrans*.[9-14] Recently, Chang et al. found novel DNA sequences with substantial sequence homology to the gammaherpesviruses Epstein–Barr virus (EBV) and herpesvirus saimiri in Kaposi's sarcoma lesions.[15,16] The putative new herpesvirus has been designated Kaposi's sarcoma–associated herpeslike virus (KSHV). KSHV sequences have been detected in tissue from more than 90 percent of HIV-1–associated Kaposi's sarcoma lesions, as well as in biopsy specimens from patients with classic Kaposi's sarcoma, endemic Kaposi's sarcoma, and post-transplantation Kaposi's sarcoma.[17-23] Two lymphoproliferative disorders related to the acquired immunodeficiency syndrome (AIDS) — body-cavity–based lymphomas[24] and multicentric Castleman's disease[25] — have also been linked to KSHV by the detection of KSHV sequences in the tumors.

To facilitate further investigation of the relation between KSHV and the pathogenesis of Kaposi's sarcoma, we have developed serologic assays for KSHV infection. The development of these assays relied on the capacity of sodium butyrate, an agent known to trigger transcription from silent cellular and viral genes,[26-28] to activate the expression of the KSHV genome in a KSHV-infected cell line.

METHODS

Patients

Serum was collected from a convenience sample of 48 patients with AIDS and Kaposi's sarcoma and 54 HIV-1–infected control patients at several clinical sites in Connecticut, New York, and California. Demographic and clinical information about the patients was recorded on standardized forms that were linked to the samples by a numerical code. Ninety-nine of the 102 patients (97 percent) were men; 92 patients (90 percent) were homosexual or bisexual (Table 1). In 46 patients the diagnosis of Kaposi's sarcoma was histologically confirmed; in the remaining 2 the diagnosis was unequivocal on clinical grounds.

Cell Lines

The BC-1 cell line was established from an AIDS-associated body-cavity B-cell lymphoma.[29,30] KSHV DNA sequences can be detected in BC-1 cells by DNA hybridization with KS330Bam and KS631Bam,

Table 1. Characteristics of the Study
Patients.*

CHARACTERISTIC	KAPOSI'S SARCOMA (N = 48)	NO KAPOSI'S SARCOMA (N = 54)
	no. of patients	
Sex		
Male	48	51
Female	0	3
Race or ethnic group		
African American	8	5
Non-Hispanic white	38	43
Hispanic	0	5
Other	2	1
Risk group		
Homosexual or bisexual	44	48
Injection drug user	0	2
Heterosexual	2	3
Other or unknown	2	1
CD4 count (cells/mm^3)		
0–100	28	21
101–300	12	16
>300	7	16
Unknown	1	1

*There were no significant differences in the characteristics shown between the patients with Kaposi's sarcoma and those without Kaposi's sarcoma by either the chi-square test or Fisher's exact test.

probes originally generated by representational difference analysis.[15,31] BC-1 cells also contain an EBV genome that is detectable with several EBV DNA probes.[30] B95-8 is an EBV-producing marmoset cell line that can be efficiently induced to express the EBV lytic-cycle gene by phorbol esters (12-O-tetradecanoylphorbol-13 acetate [TPA]).[32,33] HH514-16 is an EBV-containing cell clone, originally from a Burkitt's lymphoma, that can be induced to express the EBV lytic-cycle gene by sodium butyrate.[28,34] BL41 is an EBV-negative Burkitt's lymphoma cell line.[35] B95-8, HH514-16, and BL41 do not hybridize with the KSHV probes (data not shown). All the cell lines were cultured in RPMI 1640 medium containing 8 to 15 percent fetal-calf serum.

Immunoblot Assays

Extracts of untreated BC-1 cells and of cells treated for 48 hours with 20 ng of TPA (Calbiochem) per milliliter, 3 mM n-butyric acid, sodium salt (Sigma), or a mixture of these inducing chemicals were prepared by sonication. HH514-16 cells, treated similarly, served to control for antibody reactivity to EBV polypeptides. Each lane of 10 or 12 percent polyacrylamide gel was loaded with an extract of 250,000 cells in sodium dodecyl sulfate sample buffer; electrophoresis, transfer to nitrocellulose, and blocking with skim milk followed standard protocols.[36] Serum samples were screened at a dilution of 1:100. Antigen–antibody reactions were detected by the addition of 1.0 μCi of staphylococcal protein A labeled with iodine-125 (Amersham), and the radioautographs were exposed to film for 24 to 48 hours. The immunoblotting assays were performed and interpreted while the serum samples remained coded.

Immunofluorescence Assay

The antigens sought were present in BC-1 cells that were either untreated or treated with 3 mM n-butyrate for 48 hours. The cells were dropped onto slides that were subsequently fixed in acetone and methanol. Serum samples were tested at a dilution of 1:10, followed by a 1:30 dilution of fluorescein sheep antihuman immunoglobulin (Wellcome). The reactivity of a serum sample with untreated BC-1 cells and its reactivity with n-butyrate–treated cells were compared. When several serum samples were used, 0.5 to 2.0 percent of untreated BC-1 cells were antigen-positive. When there was reactivity with 10 to 20 times more n-butyrate–treated BC-1 cells than untreated cells, the reaction was considered to be positive. Serum samples containing antibodies to EBV but not to KSHV recognized the same number of antigen-positive cells in both untreated and n-butyrate–treated preparations. All the immunofluorescence tests were performed on coded serum samples, and the results were interpreted by readers unaware of the patients' disease status or the results of the immunoblot assays.
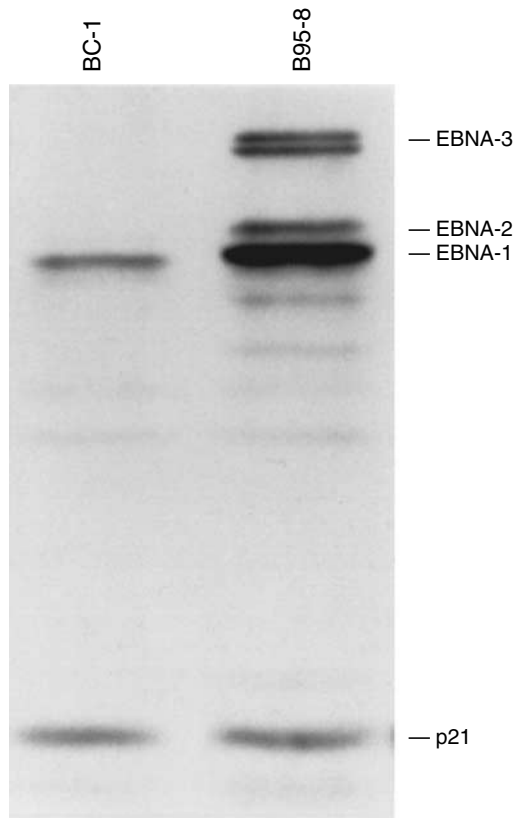
RESULTS

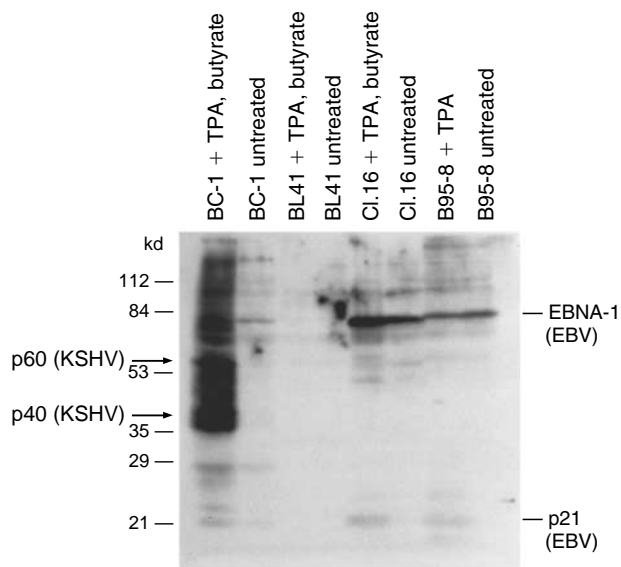### Chemical Induction of KSHV-Associated Proteins in BC-1 Cells

Because serum samples from an HIV-1–infected patient would be expected to contain antibodies to EBV polypeptides whether or not the patient had Kaposi's sarcoma and because BC-1 cells are dually infected with KSHV and EBV, it was essential to distinguish EBV polypeptides from polypeptides encoded or induced by KSHV. The technique of immunoblotting was used to determine whether the BC-1 cells expressed antigenic polypeptides specific for KSHV infection. Figure 1A shows that BC-1 cells expressed at least two EBV polypeptides, representing the latent nuclear antigen EBNA-1 and p21, a late capsid-antigen complex,[37] that were also present in other EBV-producer cell lines, such as B95-8 (Fig. 1A) and HH514-16 (Fig. 1B and 2). When serum samples from patients with Kaposi's sarcoma were used as a source of antibody in immunoblot reactions with extracts from untreated BC-1 cells, they did not identify additional antigenic polypeptides that were not also seen in the EBV-producer cell lines. However, when extracts were prepared from BC-1 cells that were first treated with a combination of TPA and n-butyrate, serum from patients with Kaposi's sarcoma recognized a number of novel polypeptides that were present in the BC-1 cell line but not in the EBV-producer cell lines (Fig. 1B and 2). The molecular weights of the most prominent of these many polypeptides were estimated at 27,000, 40,000, and 60,000 on 10 percent polyacrylamide gels. These polypeptides were detected within 24 hours after the addition of the inducing agents. Since p27, p40, and p60 were not detected in the untreated cells and appeared after the treatment with chemicals, they were thought likely to represent lytic-cycle rather than latent-cycle polypeptides of KSHV. Further experiments showed that n-butyrate was the chemical agent primarily responsible for the induction of p40 (Fig. 2).

Specificity of p40 for KSHV

Figures 1B and 2 show that no antigenic polypeptides corresponding in molecular weight to p40 were observed in two EBV-producer lines, B95-8 and HH514-16, when the EBV lytic cycle was induced by the same chemicals. Nor was p40 detected in similarly treated, EBV-negative BL41 cells. Many serum samples from patients with Kaposi's sarcoma still recognized KSHV-associated p40 when they were diluted so that they no longer reacted with EBV polypeptides (data not shown). Furthermore, n-butyrate strongly induced the expression of p40 in BC-1 cells but had little or no effect on the level of expression of the EBV

Figure 1. Immunoblots Demonstrating the Specific Recognition of KSHV Polypeptides in Chemically Treated BC-1 Cells.

Panel A shows the reactivity of untreated BC-1 cells (from a cell line established from an AIDS-associated body-cavity B-cell lymphoma) and B95-8 cells with a 1:200 dilution of a reference human antibody to EBV. The reference antibody recognizes the EBV polypeptides EBNA-1 and p21 in the BC-1 cells. Panel B shows the reactivity of various untreated and chemically treated cell lines with a 1:400 dilution of serum from a patient with Kaposi's sarcoma. The cells were treated for 48 hours with 12-O-tetradecanoylphorbol-13 acetate (TPA) and n-butyrate. The serum tested in Panel B recognizes many antigenic polypeptides in the treated BC-1 cells. For a description of the cell lines, see the Methods section. The immunoblots were prepared from 10 percent sodium dodecyl sulfate–polyacrylamide gels.

messenger RNA (mRNA) but had little or no effect on the content of EBV DNA or EBV late-cycle mRNA (data not shown). TPA, by contrast, induced the EBV lytic cycle in BC-1 cells efficiently; treatment with TPA increased the abundance of EBV DNA but caused only minimal induction of KSHV DNA.[38] These findings suggested that the switch of the two gammaherpesviruses carried by BC-1 cells from the latent to the lytic cycle was under separate control and provided further evidence that the p40 complex observed after n-butyrate treatment was specific to the KSHV genome.

Studies of p40 as a Serologic Marker for KSHV

Although a few highly reactive serum samples from patients with Kaposi's sarcoma, such as the one used in Figure 1B, recognized multiple antigenic proteins unique to the chemically treated BC-1 cells, including p27, p40, and p60, serum from other patients with Kaposi's sarcoma did not react with p27 or p60 but did recognize p40 (Fig. 2B). Therefore, recognition of p40 was studied as a serologic marker for infection with KSHV. Serum samples from the 102 HIV-1–infected patients were examined for the presence of antibodies against p40 (Table 2). Thirty-two of the 48 patients with Kaposi's sarcoma (67 percent) had such antibodies, as compared with only 7 of the 54 patients without Kaposi's sarcoma (13 percent, P<0.001 by the chi-square test). These seven patients were homosexual or bisexual men from New York City or San Francisco. None of the 13 HIV-positive patients without Kaposi's sarcoma from Connecticut had serum containing p40 antibodies. The positive and negative predictive values of this serologic marker for the presence of Kaposi's sarcoma were 82 percent and 75 percent, respectively. The patients with Kaposi's sarcoma who did not have p40 antibodies did not differ from those with p40 antibodies with regard to demographic or HIV-related variables (Table 3).

Immunofluorescence Assays

Immunoblot assays showed that n-butyrate induced the expression of KSHV lytic-cycle polypeptides in BC-1 cells without substantially affecting the expression of EBV polypeptides (Fig. 1B and 2, and unpublished data). Therefore, we reasoned that n-butyrate

early or late antigens in the same cells, which were detected with monospecific antibodies to EBV gene products. Thus, the presence of p40 appeared to represent specific expression of the KSHV genome in the chemically induced BC-1 cells. In related experiments, we have found that n-butyrate treatment also increased the abundance of KSHV DNA and KSHV late lytic-cycle
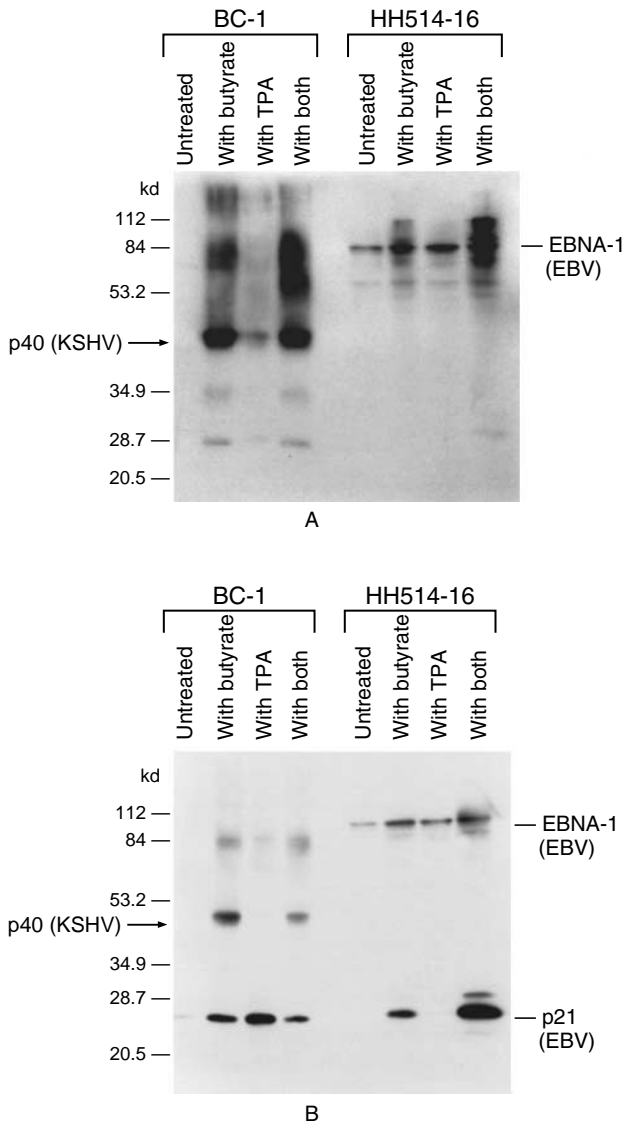
Figure 2. Immunoblots Showing the Detection of KSHV p40 by Antiserum from Patients with Kaposi's Sarcoma.

Extracts were prepared from BC-1 cells (containing KSHV and EBV) and cloned HH514-16 cells (containing only EBV) that were either untreated or treated chemically for 48 hours with *n*-butyrate, 12-*O*-tetradecanoylphorbol-13 acetate (TPA), or both. Panels A and B show the reactivity of immunoblots prepared from 12 percent sodium dodecyl sulfate–polyacrylamide gels with 1:200 dilutions of serum from two HIV-1–positive patients with Kaposi's sarcoma.

might also induce many more BC-1 cells to switch into the KSHV replicative cycle than into the EBV lytic cycle. When we performed indirect immunofluorescence assays with a reference human antiserum that contained antibodies to EBV but not to KSHV (Fig. 1A), 2 percent of cells in the untreated BC-1 line were antigen-positive, as were a similar percentage of the BC-1 cells treated with *n*-butyrate. The use of serum that was EBV-positive and highly reactive to KSHV antigens (Fig. 1B) identified 2 percent of cells in the untreated BC-1 population as antigen-positive, presumably the

EBV-expressing cells, whereas 30 to 50 percent of the BC-1 cells treated with *n*-butyrate were shown to be antigen-positive. The antigens detected were found mainly in the cytoplasm and on the cytoplasmic membrane (Fig. 3). This increased number of antigen-positive BC-1 cells in the *n*-butyrate–treated population served as the basis of an immunofluorescence assay to screen for antibodies to inducible KSHV antigens.

The results of the immunofluorescence assay were nearly identical to those of the immunoblot assay (Table 2). Sixty-five percent of patients with Kaposi's sarcoma and 13 percent of HIV-1–infected patients without Kaposi's sarcoma had serum reactive by the immunofluorescence assay. Only 3 of the 102 serum samples tested (3 percent) yielded discordant results on the two assays. One serum sample scored positive by the immunofluorescence assay and negative by the immunoblot assay; two were considered positive by the immunoblot assay and negative by the immunofluorescence assay.

The odds ratios for the association of these antibodies with Kaposi's sarcoma were 13.4 (95 percent confidence interval, 4.5 to 42) for the immunoblot assay and 12.2 (95 percent confidence interval, 4.1 to 38) for the immunofluorescence assay. The predictive value of a positive test for antibodies by either assay was 82 percent. Thus, the presence of antibodies in the serum of HIV-1–infected persons to chemically induced KSHV-associated antigens was strongly correlated with the clinical presence of Kaposi's sarcoma.

DISCUSSION

The recent discovery of genetic sequences representative of a new human herpesvirus in Kaposi's sarcoma tissue, together with past epidemiologic observations, strongly implicates this novel agent in the pathogenesis of Kaposi's sarcoma. However, these observations in themselves do not permit the construction of a unified theory of pathogenesis that accounts for the many unexplained features of Kaposi's sarcoma. Because it would allow the infection rate in various populations to be identified, a serologic marker for infection with KSHV would be a great aid in determining the role of the new virus.

Our findings, using tests for antibodies to chemically

Table 2. Prevalence of Antibody to *n*-Butyrate–Induced KSHV Antigens in HIV-1–Positive Patients with and without Kaposi's Sarcoma (KS).*

STATE OF RESIDENCE	ANTIBODY TO p40 ANTIGEN		ANTIBODY DETECTED BY THE IMMUNOFLUORES-CENCE ASSAY	
	PATIENTS WITH KS	PATIENTS WITHOUT KS	PATIENTS WITH KS	PATIENTS WITHOUT KS
	no. with antibody/no. studied (%)			
Connecticut	10/14 (71)	0/13	10/14 (71)	0/13
New York	15/23 (65)	3/28 (11)	15/23 (65)	3/28 (11)
California	7/11 (64)	4/13 (31)	6/11 (55)	4/13 (31)
All	32/48 (67)	7/54 (13)	31/48 (65)	7/54 (13)

*The odds ratios for the presence of antibody when patients with Kaposi's sarcoma were compared with patients without Kaposi's sarcoma were 13.43 (95 percent confidence interval, 4.48 to 42.0) for antibody to p40 antigen and 12.24 (95 percent confidence interval, 4.11 to 38.0) for antibody detected by the immunofluorescence assay.

induced KSHV antigens, are most consistent with a model in which KSHV infection is infrequent but is associated with a high rate of clinically apparent disease. Only 13 percent of HIV-1–infected patients without Kaposi's sarcoma had antibodies to the KSHV antigen; by contrast, a very large proportion of HIV-1–infected men with clinically evident Kaposi's sarcoma were seropositive. Further evidence for low rates of seroreactivity in patients without Kaposi's sarcoma has been provided by Moore et al. in studies of HBL-6 cells, another KSHV- and EBV-infected cell line.[38] Using serum samples from HIV-1–infected patients, they found that mean antibody titers to uninduced HBL-6 cells were nine times higher in 14 patients with Kaposi's sarcoma than in 16 patients without Kaposi's sarcoma.

Another possible interpretation of our data is that KSHV infection may be ubiquitous, but that antibodies to *n*-butyrate–induced viral antigens are not normally detectable in healthy infected persons. These antibodies might appear only after the virus has been reactivated from the latent into the lytic cycle, as might occur in the course of immunosuppression. Thus, our serologic tests might detect markers of reactivated infection but not of past exposure to the virus. If this interpretation is correct, it should be possible to demonstrate KSHV DNA sequences or to isolate the virus from healthy persons whose serum is nonreactive, either to p40 antigen or by the immunofluorescence assay.

In our study, seven patients had positive serologic tests but no clinical evidence of Kaposi's sarcoma. They were all homosexual men from New York City or San Francisco, cities whose populations are at higher risk for Kaposi's sarcoma than the general North American population.[7] Kaposi's sarcoma subsequently developed

Untreated | Treated

Kaposi's sarcoma
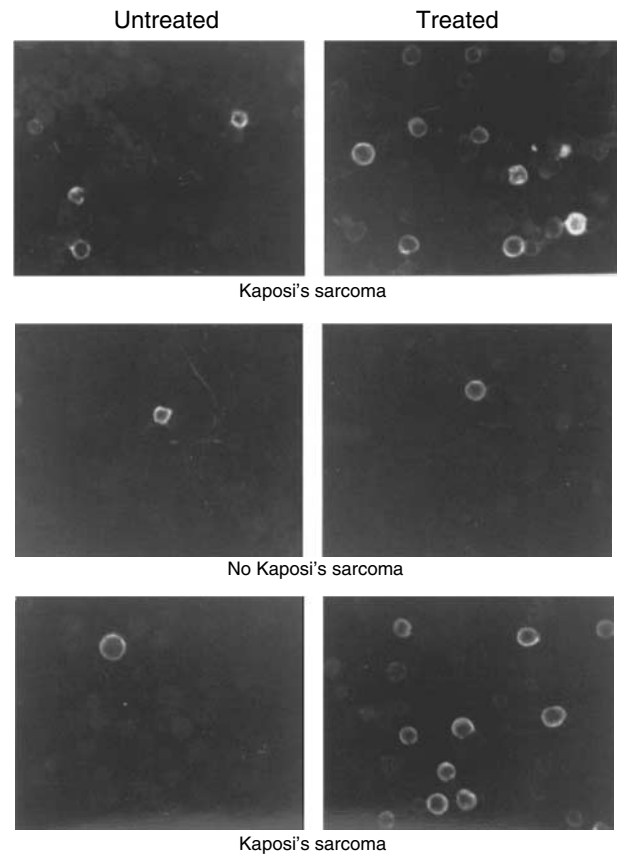
No Kaposi's sarcoma

Kaposi's sarcoma

Figure 3. Detection of KSHV Lytic-Cycle Antigens by an Indirect Immunofluorescence Assay.

BC-1 cells were either untreated (left-hand panels) or treated with *n*-butyrate for 48 hours (right-hand panels). Indirect immunofluorescence assays were performed with 1:10 dilutions of serum from each of two patients with Kaposi's sarcoma (top and bottom panels) and serum from a control patient without Kaposi's sarcoma (middle panels).

Table 3. Characteristics of Patients with Kaposi's Sarcoma, According to p40 Status.*

Characteristic	Patients with p40 (N = 32)	Patients without p40 (N = 16)
Race or ethnic group		
African American	7	1
White	25	13
Other	0	2
Risk group		
Homosexual or bisexual	29	15
Heterosexual	2	0
Other or unknown	1	1
CD4 count (cells/mm³)		
0–100	17	11
101–300	9	3
>300	6	1
Unknown	0	1
Extent of Kaposi's sarcoma†		
Limited	22	9
Extensive	10	7
Biopsy-confirmed Kaposi's sarcoma	30	16

*There were no significant differences in the characteristics shown between the patients with p40 antigen and those without p40 antigen by either the chi-square test or Fisher's exact test.

†Limited denotes disease confined to the skin or lymph nodes or with minimal mucosal involvement, and extensive denotes disease with widespread mucosal or visceral involvement or cutaneous disease with substantial edema or ulceration.

in two of the patients from New York; one had gastrointestinal involvement, and the other cutaneous lesions. Possibly the visceral lesions were present at the time of the initial evaluation.

Approximately 30 percent of the patients with Kaposi's sarcoma had no demonstrable seroreactivity in our assays. Several explanations for this are possible. The p40 antigen may not be abundant or may be only weakly antigenic in some patients. If antibody to p40 indicates the extent of lytic KSHV replication, the appearance of these antibodies may vary in different phases of the disease. It is unlikely that these patients were not infected with KSHV, since the genetic sequences are nearly universal in Kaposi's sarcoma lesions.[25] In a separate study, KSHV sequences were identified in peripheral-blood mononuclear cells, Kaposi's sarcoma tissue, or both from three of the KSHV-seronegative patients from California who had Kaposi's sarcoma.[20]

The p40 antigen is likely to be only one of a number of KSHV antigens that are recognized by the serum of infected patients. Antibody recognition of other KSHV

antigens may be impossible on immunoblot assays for several reasons: because the antigens comigrate with EBV polypeptides, because BC-1 cells cannot be induced to express these antigens, or because the antigens are not abundant or are denatured on the immunoblots. These tests using whole BC-1 cells as antigen are clearly first-generation assays, to be improved by better characterization of the KSHV gene products and by the preparation of recombinant antigens.

In summary, we describe immunoblot and immunofluorescence screening assays to detect antibodies to *n*-butyrate–induced antigens that are likely to represent lytic-cycle gene products of KSHV. Our findings support the hypothesis of a strong association between KSHV infection, as defined by the presence of antibodies to the inducible antigens, and clinically evident Kaposi's sarcoma in HIV-1–infected patients.

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