Establishment and Characterization of a Primary Effusion (Body Cavity-Based) Lymphoma Cell Line (BC-3) Harboring Kaposi’s Sarcoma-Associated Herpesvirus (KSHV/HHV-8) in the Absence of Epstein-Barr Virus

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The recently identified Kaposi’s sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), has been found to be consistently associated with an unusual subset of acquired immunodeficiency syndrome-related lymphomas, the so-called body cavity-based lymphomas (BCBL) or primary effusion lymphomas (PEL). These lymphomas are characterized by a unique spectrum of morphologic and molecular characteristics, and grow as lymphomatous effusions without an identifiable contiguous tumor mass. Until now, efforts to delineate the role of KSHV in the pathogenesis of PELs have been hampered by the lack of appropriate model systems and the concomitant presence of Epstein-Barr virus (EBV) in nearly all cases examined, and in all previously established cell lines. We now report the establishment and characterization of a novel PEL cell line, BC-3, which is KSHV+ by polymerase chain reaction (PCR) but EBV− as assessed by a variety of methods including PCR for EBER, EBNA-2, and EBNA-3C. This cell line was established from a lymphomatous effusion obtained from an HIV+ patient, and has immunophenotypic and molecular features consistent with the diagnosis of PEL, including an indeterminate immunophenotype with a B-cell immunogenotype and lack of c-myc proto-oncogene rearrangements. Pulsed-field gel electrophoresis shows an intact KSHV genome of about 170 kb both in the cell line and in the viral isolate, whereas herpesvirus-like capsids are visible by electron microscopy. Consequently, the BC-3 cell line represents an invaluable tool as a source of KSHV, for both the evaluation of the pathogenic potential of this virus and the mechanistic characterization of its role in the development of Kaposi’s sarcoma and malignant lymphoma.

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RECENT STUDIES have shown the presence of DNA sequences belonging to a novel herpesvirus in Kaposi’s sarcoma (KS) tissues. This virus has been called KS-associated herpesvirus (KSHV) and is provisionally designated human herpesvirus 8 (HHV-8). Although KSHV does not appear to be present in control tissues from non-acquired immunodeficiency syndrome (AIDS) patients or in the peripheral blood of normal blood donors, it has also been detected in two unique and distinctive categories of lymphoid proliferations, namely the primary effusion (body cavity-based) lymphomas (PEL)4 and multicentric Castleman’s disease. The PELs are a distinct group of B-cell non-Hodgkin lymphomas presenting with a unique spectrum of clinical, morphologic, immunophenotypic, and molecular genetic characteristics that distinguish them from most AIDS-related lymphomas. More specifically, PELs tend to present in the pleural, pericardial, and/or abdominal cavities as lymphomatous effusions, usually in the absence of any identifiable tumor mass throughout the clinical course. They have an unusual immunophenotype in that they commonly express CD45 in the absence of other B- or T-cell lineage-restricted antigens. At the molecular level, they are characterized by a B-cell genotype as determined by clonal Ig gene rearrangements, the presence of Epstein-Barr virus (EBV) and the lack of c-myc gene rearrangements.

Extensive sequence analyses of fragments of KSHV have shown considerable homology with Herpesvirus saimiri (HVS) and EBV. Because these two viruses belong to the gammaherpesvirus subfamily, which is characterized by its propensity to infect and transform lymphoblastoid cells, it would appear reasonable to assume that KSHV may also be a transforming agent.

In an attempt to develop in vitro model system suitable for the isolation and characterization of KSHV, our laboratory recently established two PEL cell lines, namely BC-1 and BC-2. Although very useful for several initial studies, these cell lines, as well as nearly all KSHV+ lymphomas described to date, fall short of the desired model system because of their concomitant infection with EBV. Here we report the establishment and characterization of a PEL cell line that harbors KSHV in the absence of EBV and releases viral particles containing the KSHV genome. We believe that this cell line will serve as an invaluable reagent for the characterization of the properties and functions of this novel, infectious agent.

MATERIALS AND METHODS

Pathologic samples, cell line establishment, and cell culture. The BC-3 cell line was established from a lymphomatous pleural effusion sample collected from a human immunodeficiency virus−negative (HIV−) patient previously reported in the literature. Mononuclear cells were isolated from the effusion samples by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation. Viable cells were plated at a concentration of 2 × 10^6/mL onto a layer of heterologous feeder cells as previously described. Cells were maintained in RPMI 1640 supplemented with 2 mM L-glutamine and 20% fetal bovine serum at 37°C in the presence of 5% CO₂. After approximately 4 weeks in culture, the feeder cells progressively disappeared compared with control plates.
giving way to a proliferating cell population. The cells from this well were progressively expanded, passing every 3 to 4 days, and seeded at 0.3 to 0.5 x 10^6/mL (cell viability was evaluated by Trypan blue exclusion). BC-1 and BC-2 cells, which are PEL cell lines previously established in our laboratory, were used as controls for the presence of KSHV and EBV genomes in the present studies.

Immunocytochemical analyses. The immunophenotype of the PEL and the BC-3 cell line were determined by direct and indirect immunofluorescence flow cytometry using a FACScan fluorescence activated cell sorter (Becton Dickinson Immunocytochemistry Systems, Mountain View, CA) as previously described.6,12-13 The monoclonal antibodies (MoAbs) used included BerH2 (CD30; Dako Corp, Santa Barbara, CA), B4 (CD19), B1 (CD20), B2 (CD21), CALLA (CD10; Coulter Immunology, Hialeah, FL), Leu1 (CD5), Leu14 (CD22), Leu20 (CD23), Leu9 (CD7), LeuM3 (CD14), la (HLA-DR), T4 (CD4), T8 (CD8), My10 (CD34), T11 (CD2), IL2R (CD24), ICAM (CD54), G28-5 (CD40); Becton Dickinson), T3 (CD3; United Biomedical Inc, Hauppauge, NY) and T9 (CD71), T10 (CD38; Ortho, Raritan, NJ). Anti sera to total Ig, kappa (κ) and lambda (λ) Ig light chains were obtained from Dako.

DNA extraction. Southern blot analyses. Genomic DNA was extracted by a salting-out procedure as previously described.14 Aliquots (5 μg) of genomic DNA were digested with appropriate restriction endonucleases as per the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN), size-fractionated by electrophoresis in 0.8% agarose gels, denatured (1.5 mol/L NaCl, 0.5 mol/L NaOH), neutralized (0.5 mol/L Tris pH 7.4, 3 mol/L NaCl), and transferred to nitrocellulose filters as per Southern.15 Filters were then hybridized appropriately with α-32P(dATP) random prime-labeled (Primelt II; Stratagene, La Jolla, CA) DNA probes for the Ig heavy-chain joining region (D<sub>j</sub>), the T-cell receptor β-chain, the c-myc oncogene, KSHV (KS3303Bam and KS6318Bam fragments),16 or EBV (terminal repeat region)17 as described previously.18

Polymerase chain reaction (PCR) conditions, oligonucleotide primers, and probes. Total RNA was isolated using the Tri-Resident nucleic acid extraction method (Molecular Research Center Inc, Cincinnati, OH) according to the manufacturer's instructions. To eliminate contaminating genomic DNA, the RNA samples were treated with 2 U RNase-free DNaseI (Boehringer Mannheim), with subsequent heat-inactivation of the enzyme. RNA-based PCR was performed on 1 μg RNA using the Supern枧 Reverse Transcriptase System (GIBCO-BRL, Grand Island, NY) according to the manufacturer's instructions. PCR reactions were also performed on RNA samples without the reverse transcription step to control for amplification due to genomic DNA contamination. PCR conditions, and sequences of oligonucleotides used for the amplification and hybridization of both the KSHV KS3303 Bam fragment and its flanking sequences, were as originally reported.18 Primers and probes for EBV included sets for the EBNA-2, EBNA-3C, and EBER regions, the first two allowing the accurate distinction of both types A and B EBV.18 Oligonucleotides and PCR conditions for these EBV regions were as previously published.19 All oligonucleotides used in these studies as PCR primers or probes were obtained commercially (GIBCO-BRL). PCR primers and probes for the KSHV open reading frames or ORFs20 were as follows: (1) for ORF 75 (membrane antigen homolog), 5' primer: 5'-AGGAGCGAGGAGACGGAATG-3', 3' primer: 5'-CCAGGTGGCCGCACCTCCT-3' and probe: 5'-CCAGGTGGCCGCACCTCCT-3'; (2) for ORF 74 (G-protein coupled receptor homolog), 5' primer: 5'-CGGCGGTGCTTACACGTTG-3', 3' primer: 5'-AGTCTGCTAGCATGTTGTTCC-3' and probe: 5'-CGGCGGTGCTTACACGTTG-3'; (3) for ORF 73, 5' primer: 5'-CGAGTTCCAGAAGTCTGTTG-3' and probe: 5'-CGAGTTCCAGAAGTCTGTTG-3'; (4) for ORF 72 (cyclin D homolog), 5' primer: 5'-CACCCGCTGAMECCTCGAGC-3', 3' primer: 5'-GATCCAGTCCCTCACATAGG-3' and probe: 5'-CGGCCACTCTATATGCAAACTG-3'. A primer set specific for the human β-actin cDNA (Stratagene) was used as a quantitative control. Because this primer set spans an intron, it can only amplify sequences that have derived from reverse-transcribed RNA and not from contaminating DNA, thus serving as an ideal positive control. The sequence of the β-actin internal oligonucleotide probe was: 5'-GGATGTCCACGTCAACTCT-3'. PCR reaction products (5-μL aliquots) were fractionated by electrophoresis and transferred to nitrocellulose filters as described above for genomic DNA Sources. Filters were hybridized with oligonucleotide probes for KSHV and EBV that were end-labeled with γ-32P(dATP) using T4 Polynucleotide Kinase (Boehringer Mannheim). The similar melting temperatures of the EBV oligonucleotide probes allowed their simultaneous hybridization to the nitrocellulose filter. Furthermore, direct sequencing of PCR products from the KS330 Bam region and flanking sequences for the purpose of verification was performed as previously described.16

Pulsed-field gel electrophoresis (PFGE). Cells (25 x 10^6/mL final), viral pellets from culture supernatants or cellular fractions (see below) were incubated overnight in 1% low-melting point (LMP) agarose (Bio-Rad, Richmond, CA) and 0.9% NaCl. Plugs were incubated 2 x 24 hours at 50°C in lysis buffer consisting of 0.5 mol/L EDTA, pH 8.0, 1% Sarkosy1, 1 mg/mL Proteinase K (GIBCO-BRL), washed, and stored in 0.5 mol/L EDTA at 4°C until ready for use. PFGE was performed using a CHEF-DRII System (Bio-Rad) as per the manufacturer's instructions. Samples were run on 1% PFGE-certified agarose (Bio-Rad) gels in 0.5 X Tris borate EDTA (TBE) at 200 V, ramped from 3 to 30 seconds for 23 hours at 14°C, with constant buffer recirculation using a peristaltic pump. Molecular-weight marker plugs were obtained commercially (PFGE Marker I-α Ladder; Boehringer Mannheim). Gels were transferred to nitrocellulose filters and probed successively with radiolabeled DNA probes for KSHV and EBV as described above.

Viral isolation and negative staining electron microscopy. Viral pellets were prepared as previously described.21-23 Briefly, BC-1 and BC-3 cells were collected by centrifugation for 10 minutes at 1,500 rpm, resuspended in 1:20 vol of culture medium, and snap-frozen (cell virus extract). Supernatant virus was obtained by centrifugation of a 1,400g-cleared conditioned culture medium for 2 hours at 23,000g at 4°C. Pellets were then resuspended in 1:50 vol of culture medium and snap-frozen. Both preparations (cell or supernatant virus) were then thawed, sonicated in a cup sonicator (Heat Systems, Framingdale, NY), cleared by centrifugation at 10,000g at 4°C, loaded on a phosphate-buffered saline (PBS)-25% sucrose cushion, and pelleted by overnight centrifugation at 70,000g at 4°C. Finally, viral pellets were resuspended in PBS and either embedded in agarose for pulsed-field electrophoresis or adsorbed onto parafilm/carbon-coated grids using the agar diffusion method, stained with 2% phosphotungstic acid (pH 6.8) and examined with a JEOL electron microscope (JEOL, Peabody, MA).

RESULTS

Morphologic and immunophenotypic characterization. The BC-3 cells were large and contained abundant basophilic cytoplasm, occasionally with a paranuclear halo, and large round to polygonal and sometimes pleomorphic nuclei with more prominent nucleoli. These morphologic features are consistent with their classification as a PEL. This classification is further consolidated by immunophenotypic analyses that showed that BC-3 cells express CD45, but do not express B-cell lineage-restricted antigens (CD19, CD20, CD21, CD22, κ or λ Ig light chains) or T-cell lineage-restricted antigens (CD2, CD3, CD4, CD5, CD8). There was
variable expression of activation antigens, with expression of CD30, CD38, CD54, CD71, and HLA-DR.

**Genotypic characterization.** To determine the lineage and clonality of these cells, we investigated several genomic markers by Southern hybridization. DNA from HL60 cells was used as a negative control for gene rearrangement, based on previous knowledge that this cell line has a germline configuration of the genes examined by the JH, T\(\beta\), and c-myc probes, even though the c-myc gene is amplified and gives a stronger hybridization signal. Restriction endonuclease digestion of genomic DNA and subsequent hybridization to appropriate radiolabeled probes showed that BC-3 cells have rearranged Ig heavy chain (Fig 1), as well as \(\kappa\) and \(\lambda\) light-chain genes (data not shown) and retain the germline configuration for the \(T\beta\) genes (Fig 1), indicating a clonal B-cell population. The c-myc proto-oncogene is also in its germline configuration, further consolidating classification as a PEL (Fig 1). Direct comparison of Southern hybridization analyses for Ig genes between DNA from the original tumor samples and DNA from the BC-3 cell line corroborated the tumor derivation of this cell line (data not shown).

**Presence of viral sequences in BC-3 cells.** PCR analyses on genomic DNA from the original tumor sample suggested the presence of KSHV sequences in the absence of EBV and HIV. Preliminary Southern blot hybridization analyses on genomic DNA from both the original tumor and the BC-3 cells also suggested the presence of KSHV in the absence of EBV sequences (data not shown). In addition, in situ hybridization for EBER was negative. To further evaluate the viral genomes present in the BC-3 cells, we examined the presence of KSHV and EBV sequences in these cells by PCR. We used a panel of three EBV genes, namely EBNA-3C, EBNA-2, and EBER, to detect both type A and B forms of EBV and to exclude the possibility of false negatives arising as a result of the presence of mutant EBV genomes with deleted sequences. As controls, we used two other PEL cell lines previously established in our laboratory, namely BC-1 and BC-2, both of which are known to be positive for KSHV, and EBV types B and A, respectively. PCR analyses showed that BC-3 cells, like BC-1 and BC-2, are positive for KSHV as judged by the presence of KSHV sequences in the BC-330 fragment. However, in contrast to BC-1 and BC-2 cells, BC-3 cells are negative for EBV as shown by the absence of hybridization signals with the EBNA-3C, EBNA-2, and EBER probes (Fig 2). To confirm the KSHV origin of these amplification products, we sequenced a 1,110-bp region containing the originally described 233-bp fragment as well as 492 bp upstream and 385 bp downstream flanking sequences. The sequences of the amplified fragments were identical to the previously reported KSHV sequence derived from a KS specimen, except for nine altered bases at positions 683 (T \(\rightarrow\) C), 686 (T \(\rightarrow\) C), 981 (T \(\rightarrow\) C), 1032 (C \(\rightarrow\) A), 1033 (C \(\rightarrow\) T), 1055 (G \(\rightarrow\) T), 1132 (A \(\rightarrow\) G), 1139 (A \(\rightarrow\) C), and 1514 (G \(\rightarrow\) A), which fall within the variation expected among different isolates. BC-3 cells are also negative for other herpesviruses such as herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and cytomegalovirus (CMV) as determined by immunohistochemistry analyses.

**Expression of KSHV sequences in BC-3 cells.** In an attempt to characterize the biologic significance of the presence of KSHV sequences in the BC-3 cell line, we used an RNA-based PCR assay to analyze the expression of four KSHV ORFs previously identified in the region surrounding the originally reported KS631 Bam fragment. These ORFs include a cyclin D homolog, a G-protein coupled receptor homolog, a homolog of HVS ORF 75 which is thought to encode a tegument protein, and the positional homolog of...
Fig 2. PCR analyses for KSHV and EBV sequences in the BC-3 cell line. (A) PCR was performed as described in the Materials and Methods, and amplified fragments were transferred to nitrocellulose filters and hybridized with an internal oligonucleotide probe for the 233-bp KSHV-330 fragment. BC-3 cells are shown to be positive for the KSHV-330 sequence as judged by comparing to the BC-1 and BC-2 cell lines (positive controls). (B) PCR for the EBV-3C, EBER, and EBNA-2 genes of EBV detected their presence in the BC-1 and BC-2 cell lines but not in the BC-3 cell line. Different primer sets allowed the discrimination between the EBNA-3C genes in type B EBV (246-bp fragment) and type A EBV (153-bp fragment) present in the BC-1 and BC-2 cell lines, respectively.

HVS ORF 73 whose function is unknown. All four ORFs were found to be expressed as determined by RNA-based PCR analyses (Fig 3), suggesting that these KSHV sequences are part of a functional viral genome rather than spurious DNA sequences. PCR on RNA samples lacking the reverse transcription step failed to yield any amplification products, thus confirming that the observed fragments did not arise from genomic DNA contamination (data not shown).

Characterization of KSHV particles in BC-3 cells. To show the presence of KSHV particles in the BC-3 cells, we analyzed the genomic DNA content by PFGE. As before, the BC-1 and BC-2 cell lines provided useful controls for the presence of KSHV and EBV genomes. As shown in Fig 4B, the BC-1 cells when hybridized with a KSHV probe show a band at about 270 kb, as previously reported. In contrast, the BC-2 and BC-3 cells show bands of approximately 200 and 170 kb, respectively. This difference is highly reproducible, and does not stem from technical or experimental variables. Viral pellets obtained from BC-3 cellular or supernatant fractions show a band of 170 kb, at the same position as that of the bands in the BC-3 cells (Fig 4C). As expected, a band at the appropriate molecular weight was identified with an EBV probe in the P3HR1 and Raji control cell lines and in the BC-1 and BC-2 cell lines, but was absent from the BC-3 cell line (Fig 4A).

Previous attempts to directly visualize KSHV particles have been obscured by the concomitant presence of EBV in all biologic samples and established cell lines thus far examined. However, the BC-3 cell line is ideally suited for the examination of KSHV particles at the electron microscopic level. Negative staining electron microscopy showed the presence of viral capsids of approximately 100 to 150 nm diameter (Fig 5). Although the two capsids shown are collapsed, ring-shaped capsomers of approximately 9-nm diameter arranged in linear arrays are discernible. Some of these capsomers clearly show up as hexons whereas others in the periphery appear as hollow tubes giving a castellated edge. These features are characteristic of herpesviruses and herpesviral preparations. Taken together with the PFGE analyses, these results indicate that BC-3 cells are infected with KSHV and release KSHV particles.

DISCUSSION

In this report we describe the establishment and characterization of a cell line, BC-3, which is shown by immunophe-
notypic and genotypic analyses to belong to a recently recognized rare group of malignancies termed PEL or primary effusion lymphomas. BC-3 cells are of B-cell origin as judged by their clonal Ig heavy- and light-chain gene rearrangements and germline T-cell receptor genes. In line with all previously published cases of PEL, the BC-3 cells are positive for KSHV sequences; however, the novelty of this cell line lies in the absence of EBV coinfection as determined by Southern hybridization, in situ hybridization, PCR, and PFGE analyses. This cell line consequently provides us an opportunity to isolate, characterize, and functionally dissect the recently discovered KSHV.

The first report of the KSHV sequences as detected in KS tissues raised questions regarding the nature of these sequences and the evidence for a bona fide viral particle. Sequencing and formal phylogenetic analyses of large portions of the KSHV genome have demonstrated that it is a γ-2 herpesvirus (genus Rhadinovirus) and the first member of this group to infect humans. Furthermore, with the establishment of the BC-1 and BC-2 cell lines in our laboratory, it became possible to visualize the KSHV DNA by PFGE analyses as well as to perform transmissibility studies to prove that these sequences belong to a functional virus capable of infecting cells and replicating in them. According to these studies, the KSHV genome in the BC-1 cell line has a molecular weight of approximately 270 kb. The data presented in this report confirm these findings, yet also show KSHV particles of lower molecular weight in the BC-2 and BC-3 cell lines (Fig 4). The molecular weight of the KSHV genomes in the BC-3 cells and viral isolates is approximately 170 kb, and this is a highly reproducible finding not attributable to technical or experimental variations. Because the viral preparations were extensively sonicated before PFGE
and Southern analyses, the fact that we obtained sharp bands corresponding to intact genomes for BC-1 and BC-3 viral isolates is an indication that these genomes are protected from sonication, and therefore evidence for their encapsidation. Moreover, KSHV DNA fragments are protected from DNase degradation, further suggesting that these viral isolates contain encapsidated genomes. (E.A.M., unpublished data, 1995). One possible explanation for the observed differences could involve dimerization or other rearrangement events of the viral genome. It is also tempting to speculate that the KSHV genome present in BC-3 cells may be a deletion mutant of the larger KSHV genomes found in BC-1 cells, in a manner analogous to the various known deletion mutants of EBV. It is interesting to note that functional differences may accompany these differences in genomic size. Viral isolates obtained from the BC-1 cell lines are capable of infecting umbilical cord blood B cells. Furthermore, KSHV transmission is blocked by UV-irradiation and foscarnet (an inhibitor of viral DNA-polymerase), thus providing evidence for the presence of a biologically active virus. Similarly, viral isolates from BC-3 cells appear to infect cell lines of the B-cell lineage (E.A.M., unpublished data, 1995). Studies to characterize the molecular and functional differences between KSHV derived from the BC-1 and BC-3 cell lines are currently underway in our laboratory.

Although the precise explanation and significance of the differences in genomic size among various KSHV isolates can only be addressed when a physical map of the virus becomes available, these particles do correspond to KSHV based on the presence and sequence analyses of the KS330 Bam fragment, which is thus far the single available criterion for detecting the presence of this virus. Further evidence that the sequences present in BC-3 cells correspond to KSHV is provided by our RNA-based PCR assays, which detected and amplified fragments from four different ORFs derived from a region very distant from the KS330 Bam fragment. Expression of these four ORFs further suggests that this virus is functional and has an active biologic role in these PEL cells. The homologies of these ORFs to genes involved in cell cycle regulation raise interesting questions as to their potential role(s) in viral transformation and/or pathogenesis. For example, ORF 72, a cyclin D homologue, may be involved in the subversion of the host cell cycle to the advantage of viral replication. In this respect, HSV contains an ORF that encodes a viral cyclin D homologue capable of interacting with host-cell cyclin-dependent kinases and thus potentially playing a role in the oncogenic transformation process. Although EBV lacks a cyclin D homologue, its latent membrane protein-1 (LMP-1) has been shown to upregulate the expression of cellular cyclin D2 leading to the loss of transforming growth factor-α-mediated growth inhibition and perhaps contributing to cellular transformation. We are currently investigating the functional characteristics of these ORFs with a view to assessing their role (if any) in KSHV-mediated transformation events.

Finally, electron microscopic examination of the viral preparations showed the presence of viral particles having the morphologic features typical of herpesviral capsids (Fig 5), and particularly those of gammaherpesviruses, suggesting that they may correspond to KSHV. These results are in agreement with transmission electron microscopy studies performed on KSHV + cells obtained from the same patient as the BC-3 cells but after passage in BNX mice. Besides being an invaluable tool for the isolation and characterization of KSHV, the BC-3 cell line provides us an opportunity to investigate the pathogenesis of PEL. An expanded cyto genetic and immunophenotypic analysis of these cells may yield clues as to the nature of the B lymphocyte which is infected by KSHV and perhaps point to the cell surface molecule(s) acting as the viral receptors. Studies involving the administration of BC-3 cells to immunodeficient (nude) mice are currently underway in our laboratory, in an effort to determine whether KSHV dictates the development and characteristics of these rare lymphomas. In summary, BC-3 cells will serve as a tool which will allow us to address the more significant questions regarding the role(s) of KSHV in the pathogenesis of PEL, KS, and multicentric Castleman’s disease.

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