CONCISE REPORT

Clonal Analysis of Transplant-Associated Lymphoproliferations Based on the Structure of the Genomic Termini of the Epstein-Barr Virus

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The clonal composition of various transplant-associated lymphoproliferations was assessed by means of Southern blot hybridizations using a DNA probe specific for the fused termini of the Epstein-Barr virus (EBV) genome. A single clonal band representing the joined EBV genomic termini was detected in most biopsies, demonstrating the presence of a monoclonal expansion of B lymphocytes carrying EBV DNA. Different configurations of immunoglobulin gene rearrangements and fused EBV genomic termini were frequently observed in tissues from different biopsy sites in individual patients, confirming the multiclonal origins for these lymphomas. In rare specimens, multiple forms of the joined termini were detected within individual lesions.

IMMunosuppressed organ-transplant recipients are susceptible to a markedly increased risk for development of unregulated B-lymphocyte proliferations. Cells in virtually all of these proliferations have been shown to contain Epstein-Barr viral genomes or antigens. The frequent association of transplant lymphoproliferations with Epstein-Barr virus (EBV) has been of unknown significance, since it could either be due to a direct role of the virus in the induction of B-cell proliferation or due to secondary infection of proliferating B cells following reactivation of latent EBV infection that invariably occurs after iatrogenic immunosuppression. The pathologic nature of these proliferations has also been unclear in the past, since it has been uncertain whether they represent true neoplastic processes or virus-associated hyperplasias. To investigate this issue several studies have focused on the clonality of the cells comprising transplant-associated lymphoproliferations, the most informative of which have used the configuration of rearranged immunoglobulin genes as clonal markers. However, the latter studies may have been complicated by the possibility that configurations of rearranged Ig genes are not completely stable clonal markers, as has recently been demonstrated in non-Hodgkin's lymphomas.

An alternative clonal marker for EBV-associated malignancies that relies on the configuration of fused termini of the EBV genome has recently been described. This method is based on the fact that the linear DNA termini of the encapsulated viral genome are joined intracellularly to form covalently closed episomal DNA following viral infection of host cells. Because there are variable numbers of tandem repeated sequences at each linear terminus, the precise molecular configuration of fused termini after circularization varies for each independently circularized genome. Multiple identical viral episomes with the same fused termini are maintained in the progeny of each infected cell. Differences in the configurations of EBV-fused termini can be detected as differently sized fragments on Southern blot analyses. If the original in vivo multiplicity of infection was low, only one circular form of EBV genome will be present in clonal infected cells, as reflected by a single fused terminal fragment detected in the tissues.

In the present study the investigators have examined the clonal composition of transplant-associated lymphoproliferations using a marker that relies on the structure of episomal EBV DNA genomes present in the proliferating B lymphocytes. The investigators' studies confirm that there is a spectrum of EBV-associated disorders of varying clonal composition that may arise in immunosuppressed organ-allograft recipients. The data are consistent with the proposal that the lymphoproliferations initiate as clonal expansions of EBV-carrying B cells, which progress to multiclonal lymphomas in most patients. Detection of homogeneous episomal EBV DNA in most lesions supports a primary role for the virus in the pathogenesis of these disorders.

MATERIALS AND METHODS

Tissue specimens serving as a source of DNA were obtained by surgical or autopsy biopsies of transplant-associated lymphoproliferations, which have been described in detail previously. DNA extracted from tissue specimens was digested with the BamHI restriction enzyme; the resulting fragments were size fractionated by agarose-gel electrophoresis, transferred to nylon membranes, and hybridized to radiolabeled DNA probes, as described earlier. Structures of immunoglobulin heavy- and light-chain gene probes have been presented elsewhere. The DNA probe for the EBV genomic termini consisted of a 5.2-kb BamHI-EcoRI fragment isolated from the fused BamHI terminal fragment NJet described elsewhere.

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Supported by grants from the Lucille P. Markey Charitable Trust and CA-42971 and CA-38621 from the National Institutes of Health, and a General Clinical Research Grant (RR-00188). Dr Michael L. Cleary is a Scholar of the Lucille P. Markey Charitable Trust. Dr Jeffrey Sklar is a recipient of Research Career Development Award from the National Institutes of Health.

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RESULTS AND DISCUSSION

DNA was isolated from tissue biopsy specimens of lymphoproliferative disorders that developed in the setting of immunosuppression for prevention of organ allograft rejection. The purified DNA was digested with the *Bam*HI restriction enzyme and subjected to Southern blot analysis using a DNA probe specific for the fused termini of the EBV genome. When DNA from tissue specimens removed from several cardiac transplant patients was examined, a single predominant band was observed in each sample (Fig 1, patients 1, 2, and 3). Similar results were observed for DNA samples isolated from lesions that developed in liver, heart/lung, kidney, and thymus recipients (Fig 1, patients 5 through 7). Detection of one predominant band with the EBV terminus probe showed that each lesion contained significant amounts of homogeneous episomal EBV DNA resulting from a single circularization event. Additional, less intense bands migrating in different positions were observed in some of these DNA specimens, showing that in addition to the predominant clonal species of EBV genome present in the tissues, minor clonal populations were also present (see below). When the same DNA specimens were analyzed previously with an IgH probe, one or two rearranged bands were observed in each lane, confirming the presence of a predominantly monoclonal B-cell population.4

![Fig 1. Analysis of EBV termini in various transplant-associated lymphoproliferations. DNA was extracted from biopsy tissue specimens derived from cardiac (patients 1 through 3), liver (patient 4), heart/lung (patient 5), kidney (patient 6), and thymic (patient 7) transplant recipients who developed lymphoproliferative disorders. After digestion with the *Bam*HI restriction enzyme, the DNAs were analyzed for configurations of EBV terminal DNA fragments by the Southern blot hybridization procedure using a probe specific for the fused termini of the virus genome. The numbers above the gel lanes refer to individual patients, and the letters refer to separate biopsy specimens from each numbered patient. Dashes to the left of gel lanes correspond to the positions of DNA size standards with sizes shown in kilobases.](image)

A similar analysis was carried out on DNA from tissue samples obtained from different biopsy sites or sequentially throughout the clinical course of each patient’s disease (Fig 1, patients 1 through 4). Within the DNA from each biopsy specimen an intense clonal band was observed following hybridization with the DNA probe specific for the fused genomic termini of EBV. However, the positions of the bands varied from site to site within a given patient. If the same clone of B lymphocytes had occupied all biopsy sites, the positions of the EBV DNA terminal bands would have been identical in every specimen from the same patient. Although identical patterns were observed occasionally at two or more sites, in most cases for which DNA could be studied from multiple biopsy sites a different configuration for the fused EBV termini was observed along with different configurations for the heavy- and light-chain Ig gene rearrangements, as described previously.4 There appeared to be no correlation between the size or intensity of the EBV terminal DNA bands and the biological or clinical behavior of the lesions. The EBV and IgH results on these tissue samples are mutually consistent and indicate that within each patient several separate clones of B lymphocytes had expanded sufficiently at different sites to be detected using the Southern blot hybridization technique. As has been suggested earlier based on Ig gene rearrangement results alone, the EBV data presented here confirm that the lymphoproliferative disorders that develop in the setting of iatrogenic immunosuppression appear to be multiclonal in origin, in contrast to the monoclonality of the vast majority of lymphomas developing in nonimmunosuppressed hosts.

A more complex clonal composition was observed when multiple tissue biopsy specimens were examined from a severe combined immunodeficiency syndrome (SCID) patient who had developed a disseminated lymphoproliferative disorder following bone marrow transplantation (BMT).4 Analysis of DNA from most of the tissues obtained from this patient did not show a single predominant band using the probe for the EBV-fused termini (Fig 2 upper, lanes 1 through 7); instead, multiple, closely spaced bands of varying intensity were observed, likely corresponding to an oligoclonal B-cell proliferation. Two sites, however, did show intense clonal bands: in the lung lesion (lane 2) an intense clonal band was observed superimposed on a background of multiple faint bands, and in the GI lesion (lane 5) three intense bands were observed in the absence of a background pattern. These results were very similar to those observed using an IgH probe (Fig 2, lower) in which monoclonal IgH rearrangements were found in the gastrointestinal (GI) and lung lesions, with a background pattern of multiple faint bands also present in the latter. These data indicate that in most of the lesions from this patient, no single clonal population was predominant, but rather a number of different minor B-cell clones were present in the tissues. In the lung lesion one B-cell clone had predominated, based on both the Ig gene rearrangement pattern and the single intense EBV band. In contrast, the GI lesion appeared to contain either a monoclonal population of B cells (with both IgH alleles rearranged) or two clonal B cell populations with a single rearranged IgH gene in each. The presence of a
germline band indicated that there were some nonlymphoid cells in the GI specimen, but the absence of faint background bands showed that, unlike all the other sites, there were no minor clones in this lesion. The presence of three EBV bands and no background bands for viral DNA suggested that three distinct species of EBV genomes were present in the B-cell clone(s) at this site, possibly reflecting a high multiplicity of infection secondary to high viral titers in a patient severely immunocompromised for both B- and T-cell responses.

The background oligoclonal pattern appears not to be unique to this particular SCID patient’s lymphoproliferations, since it has been observed in tissue biopsy specimens removed from other patients. For example, the pattern of EBV-fused terminal fragments shown in Fig 2 (lanes 8 and 9) were obtained from left and right tonsillar biopsies performed on a cardiac transplant patient. In each tonsil a predominant clonal band is superimposed on a background of multiple faint bands. Since the migration of the predominant monoclonal EBV-fused terminal fragment is different at the two sites, it indicates that unrelated B-cell clonal proliferations had developed within each lesion. Analysis of these DNA specimens with the IgH probe (Fig 2, lower) showed an ill-defined faint band in each specimen. Since the investigators have found to be nonspecific and frequently associated with reactive lymph node processes (Cleary et al, unpublished observations), it is possible that these bands may represent positions of standards with sizes in kilobases. Dashes in the lower portion of this figure refer to germline configurations of the IgH gene.

The above data indicate that in some lesions monoclonal and oligoclonal B-cell populations may coexist at the same site. In the SCID patient, results from the various biopsy sites likely represent different states in the development of these lymphoproliferations. The multiple faint bands may correspond to the initial emergence of dominant B-cell clones from a polyclonal collection of proliferating B cells. From this oligoclonal proliferation a dominant monoclonal population of B cells may eventually emerge, and with time this clone completely outgrows and obscures the remaining B cells. The SCID patient appears unusual in that most of the biopsies reflect the early stages of this pathogenetic process. In the investigators’ experience biopsy specimens from immunosuppressed organ transplant recipients typically reflect the later stages of this process in that they contain a single dominant B-cell population; however, as our above results demonstrate, a similar pathogenesis may underlie all lesions, since rare biopsy specimens from non-SCID transplant patients also contain the oligoclonal pattern. Detection of minor, faint EBV bands in the lesions is consistent with this proposal and possibly reflective of minor clones that occasionally and transiently coexist with the dominant clone.

The molecular basis for the use of immunoglobulin gene rearrangements as clonal markers in human lymphoid disorders has been described in detail previously. A potential disadvantage of using Ig gene rearrangements as clonal markers is that these genes are subject to somatic hypermutation, which recent studies have demonstrated can occur in non-Hodgkin’s lymphomas. In addition, in murine tumor-cell lines, differences in Ig gene rearrangement configurations may result from V-region substitution rearrangements within previously rearranged immunoglobulin genes. Since the transplant-associated lymphoproliferations most closely resemble non-Hodgkin’s lymphomas pathologically, a potential criticism of earlier clonal studies using Ig probes in these disorders concerns the stability of rearranged configurations of Ig genes in transplant-associated lymphoproliferations, particularly in light of the results indicating a highly unusual multiclonal composition for these lesions. The DNA composing EBV-fused termini, on the other hand, would not be expected to undergo somatic hypermutation and in fact appear to be quite stable following circularization of the viral genome after infection of the host B lymphocyte. The configuration of EBV-fused termini thus represents a useful and potentially more reliable clonal
marker in B-cell lymphomas showing a frequent EBV association, such as those examined in this study; and the data presented here using a probe for the EBV termini support our previous Ig gene data demonstrating the multiclonality of transplant-associated lymphoproliferations.5'6

The investigators’ data also support a primary role for viral involvement in the pathogenesis of these lymphoproliferations. In the proliferations that typed as monoclonal, the fact that all of the tumor cells were infected with a single form of the EBV genome indicates that the B-cell proliferations probably occurred after EBV infection. The investigators’ results suggest that the mechanism for development of monoclonal lesions may depend on the ability of B lymphocytes latently infected with EBV to proliferate in an uncontrolled manner under conditions of reduced immunosurveillance. Additional genetic events may occur in certain members of a polyclonal B-cell proliferation, resulting in a relative growth advantage and the eventual emergence of a predominant monoclonal population. The additional events required for transition to a monoclonal tumor may take the form of karyotypic abnormalities, which have been described in some transplant-associated tumors.7,15

ACKNOWLEDGMENT

We thank Eileen Gunther for assistance in preparation of the manuscript, Phil Verzola for photographic assistance, and Jessica Spies and Diana Stocks for technical support. We thank Dr M. Calos for making available an EBV DNA library from which the fused terminus probe was isolated.

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