Clonal Origins of Lymphoproliferative Disease Induced by Epstein-Barr Virus

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Analysis of immunoglobulin gene rearrangements in 16 B-cell lineages clonally propagated from two mononucleosis patients supported the notion that mononucleosis is a polyclonal B-lymphoproliferative disorder. Three of seven cell clones from a patient with a fatal B lymphoma revealed the same pattern of immunoglobulin gene rearrangement, indicating that this patient's disease was oligoclonal. The three similar clones were propagated from two sites (blood and spleen), indicating that they represent a metastatic cell lineage which arose during the patient's fatal B lymphoproliferation.

The Epstein-Barr virus (EBV) exhibits a tropism for B lymphocytes and can transform them in vitro into long-term cell lines. Studies of EBV-transformed B cells from patients with infectious mononucleosis have suggested that EBV infection in these patients is a polyclonal process (1, 8, 12). However, analysis of the clonal origin of EBV-induced lymphoproliferative disease is confounded by the fact that cells with one phenotype—e.g., cells producing immunoglobulin M [IgM(κ)]—are the preponderant phenotype in most B-cell populations. Furthermore, B cells producing similar heavy- and light-chain isotypes may comprise a heterogeneous cell population, with individual cells producing antibodies of different specificities, resulting from variations in chromosomal rearrangement and somatic mutation at the immunoglobulin heavy- and light-chain gene loci (15). Thus, it is difficult to establish definitively the polyclonal, oligoclonal, or monoclonal origin of a B-cell population by analysis of immunoglobulin isotype production. Idiotypic analysis can shed further light on this issue, but is confounded by technical difficulties. In this study we have utilized direct cell cloning and molecular genetic analyses of immunoglobulin gene rearrangements to assess the clonal origin of EBV-related B-lymphoproliferative disease in two patients with infectious mononucleosis and one patient with a fatal, EBV-associated B lymphoma.

Materials and methods. Derivation of the cell clones from the patients has been previously described (1, 11). In this paper we emphasize that all of the cell clones were derived by direct plating of fresh lymphocytes in soft-agar medium, without prior in vitro cultivation (1, 11). These clonally derived B-cell lines were propagated in RPMI 1640 with 10% fetal bovine serum and antibiotics (penicillin, 50 IU/ml; streptomycin, 50 μ g/ml; and amphotericin, 1 μ g/ml). DNA was extracted from washed cell pellets of $\geq 10^8$ cells by standard methods (7). DNA samples were digested with 4 to 5 U of restriction enzyme per μ g of DNA for 4 to 5 h at 37°C, under the buffer conditions recommended by the supplier (Bethesda Research Laboratories). DNA fragments were electrophoresed in 0.6% agarose gels and blotted to nitrocellulose paper by the Southern method (14). The immunoglobulin gene probes used for hybridizations with the Southern blots were described previously (2, 6). Probe DNAs were

labeled by nick translation with [³²P]dCTP to specific activities of $\geq 1 \times 10^8$ cpm/ μ g (10). Hybridization and washing conditions were by the method of Wahl et al. (16). Hybridized blots were exposed to Kodak XAR-5 film for 2 to 10 days, and intensifying screens were used.

Clonal origin of the transformed cells propagated from mononucleosis patients. The sets of cell clones from the two mononucleosis patients were labeled IM-F and GG57 (Fig. 1). Each set comprised isogenic clones derived from one blood sample taken from the designated patient in the acute stage of mononucleosis (1). The clone designations are indicated above the lanes. The blots in Fig. 1A and B were hybridized with a probe for the immunoglobulin heavy-chain joining segment (J_H). Each cell clone shows two bands of strong hybridization with the J_H probe, corresponding to the two allelic J_H loci in each clone. The configuration of the two major J_H bands is different for each clone, reflecting the clonal uniqueness of chromosomal joinings of heavy-chain variable regions (V_H), diversity segments (D), and J_H segments, i.e., the uniqueness of V_H-D-J_H joinings in each cell clone (15).

One feature of note in Fig. 1B is the anomalous J_H hybridization result for cell clone GG57-17. This clone exhibits one major J_H band at 16.3 kilobases (kb) and two smaller bands at 5.4 and 4.7 kb. This clone has been repeatedly found by cytoplasmic immunofluorescence to produce only IgG(λ) and appears to have a single rearranged λ light-chain locus on hybridization analysis (radiogram not shown). Thus, we feel that this cell line is probably truly clonal, and it appears likely that this clone contains a duplication of a chromosomal locus containing J_H-like sequences or that one J_H locus has acquired an intra-allelic *Bam*HI site, although these alternatives can be firmly established only by molecular cloning data.

Clonal origin of the transformed cells propagated from the lymphoma patient. Initial analysis of *Bam*HI-digested DNAs from the seven cell clones derived from the lymphoma patient suggested that three of the cell clones (TS-L3, TS-S2, and TS-S4) had a similar pattern of J_H rearrangement; these three clones had previously been shown to produce IgM(κ) (11). The other four clones produce different immunoglobulin isotypes and were found to have different J_H rearrangements. The TS-L3, TS-S2, and TS-S4 clones were derived as independent colonies of EBV-transformed lymphocytes

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FIG. 1. Southern hybridization analysis of immunoglobulin J_H gene rearrangements in *Bam*HI digests of DNAs from B-cell clones derived from patients IM-F (A) and GG57 (B), both with infectious mononucleosis. Cell clones IM-F35, IM-F9, IM-F11, GG57-9, GG57-15, and GG57-20 produce IgM. Cell clones IM-F17, IM-F2, IM-F5, IM-F19, IM-F32, GG57-5, GG57-14, and GG57-17 produce IgG. Cell clones IM-F26 and GG57-16 produce IgA. Similar data for three other IgM-producing IM-F cell clones are not depicted here. Dark arrow at left margin indicates the 16.3-kb-pair *Bam*HI blot position of the J_H gene in germ line DNA. Numbers at left indicate approximate fragment sizes, in kilobases, and are derived from a plasmid marker lane on each blot. (A) The second J_H allele (5.0 kb) for cell clone IM-F32 has been cut off, as doing so allowed clearer illustration of the other J_H bands in the figure, which are all >11 kb. The results in panels A and B indicate that the various cell clones from the two mononucleosis patients have distinct patterns of J_H gene rearrangement. The two cell clones with the closest similarity, IM-F35 and IM-F5, have previously been shown to produce different immunoglobulin heavy chains and to have different rearrange-

ments of heavy-chain constant-region sequences (2). (B) Each of the wide, dark bands of J_H hybridization for cell clones GG57-9 and GG57-16 represents a doublet, corresponding to rearranged J_H alleles which are close in size in *Bam*HI digests. A band of lighter J_H hybridization, visible in panel B at 10.7 kb, is due to the presence of other sequences in human DNA which are partially homologous with J_H and which are detected whenever our probe is labeled to $\geq 3 \times 10^8$ cpm/ μ g. The anomalous result for cell clone GG57-17 is discussed in the text.

from mononuclear cells of blood (TS-L3) or spleen (TS-S2 and TS-S4) (11). The similarity of immunoglobulin gene rearrangement in these three cell clones was subsequently confirmed as follows. First, we analyzed *Bam*HI digests of DNAs extracted from several different frozen samples of each cell line, confirming consistent results (radiograms not shown). Second, we cut the TS-L3, TS-S2, and TS-S4 DNAs with three restriction enzymes (Fig. 2). Hybridization with the J_H probe revealed identical *Bam*HI, *Eco*RI, and *Hind*III fragments for all three cell clones, except that TS-S4 consistently has a 7.6-kb, J_H -hybridizing *Bam*HI fragment that is not seen in the other two cell clones (Fig. 2A). This extra fragment is consistently present in the DNA extracted from three frozen samples of this cell line. It is not due to plasmid contamination of the DNA(s), since it is not seen with other pBR-based probes or with nick-translated pBR322 DNA. Since the TS-L3, TS-S2, and TS-S4 clones appear identical in J_H hybridizations of *Eco*RI and *Hind*III digests, the most likely explanation for the extra, 7.6-kb, J_H -hybridizing *Bam*HI fragment seen in TS-S4 is that TS-S4 has a duplication of a chromosomal region containing J_H sequences of one of this clone's J_H alleles, whereby the duplicated segment has preserved the normal *Eco*RI and *Hind*III sites flanking the J_H sequences of the corresponding J_H allele. This probable gene duplication event is cytogenetically occult, since repeat analysis of the G-banded chromosomes from TS-S4 reveals a normal female karyotype (46, XX).

The DNAs of cell clones TS-L3, TS-S2, and TS-S4 were also found to have the same pattern of κ light-chain gene rearrangement (Fig. 2B). This finding provides additional confirmation of the sibling relationship of the clones as independent clonal descendants of the same B-cell lineage.

Discussion. The analysis of J_H rearrangements in the cell clones from the mononucleosis patients supported the notion that mononucleosis is a truly polyclonal B lymphoproliferation. By direct cell cloning and genetic analysis of 16 cell clones, we found no evidence of oligoclonal origin of the disease process in two mononucleosis patients.

The lymphoma patient's disease began as a mononucleosislike illness and was associated with serologic evidence of primary EBV infection (11). This disease progressed rapidly, however, with unchecked proliferation of EBV-infected B cells. At autopsy, the histologic picture was that of diffuse, undifferentiated B lymphoma, which was reported as a polyclonal disease on the basis of phenotypic analyses of cells in blood and tissue sections, as well as similar analyses of the presently studied cell clones (11). We did not suspect, at that time, that the three IgM(κ)-producing cell clones were sibling descendants of the same B-cell lineage, as has been revealed by the present analyses of immunoglobulin heavy- and light-chain gene rearrangements. The present results suggest that this lymphomatous process was becoming oligoclonal, within a week after onset of illness. Evidence suggesting that malignant, EBV-related B lymphoproliferations in immunodeficient or immunosuppressed patients

ments of heavy-chain constant-region sequences (2). (B) Each of the wide, dark bands of J_H hybridization for cell clones GG57-9 and GG57-16 represents a doublet, corresponding to rearranged J_H alleles which are close in size in *Bam*HI digests. A band of lighter J_H hybridization, visible in panel B at 10.7 kb, is due to the presence of other sequences in human DNA which are partially homologous with J_H and which are detected whenever our probe is labeled to $\geq 3 \times 10^8$ cpm/ μ g. The anomalous result for cell clone GG57-17 is discussed in the text.

FIG. 2. Southern hybridization analyses of the TS-L3, TS-S2, and TS-S4 cell clone DNAs. (A) Cell clone DNAs were digested with *Bam*HI, *Eco*RI, or *Hind*III, blotted, and hybridized with the immunoglobulin J_H probe. The three cell clones have the same pattern of J_H hybridization, except that TS-S4 consistently exhibits a 7.6-kb band of hybridization with the J_H probe in *Bam*HI digests only, a result which is not seen in the other two cell clones (see text for details). HPC is a pooled human placental DNA which was utilized to indicate the germ line positions for unarranged J_H fragments with each of the three endonucleases. The germ line position for the J_H gene in *Bam*HI digests (16.3 kb) is indicated by a dark arrow at the left. The human placental DNA always gives a faint signal, due to partial degradation of this commercially supplied DNA. Nevertheless, it clearly indicates the J_H germ line position at 9.6 kb in *Hind*III digests. Comparison of this result with the *Hind*III results for the three TS cell clones indicates that the cell lineage represented by the TS clones had biallelic J_H rearrangements, a point which was equivocal in the *Bam*HI and *Eco*RI digests. (B) *Bam*HI and *Eco*RI digests of the DNAs from TS-L3, TS-S2, and TS-S4, hybridized with a probe for the κ light-chain constant region (C_κ). The κ light-chain gene rearrangements in the three TS clones appear to be the same, as is evident in the results for the *Bam*HI digests. *Eco*RI digests indicate a normal-sized (~2.5 kb) fragment containing C_κ in patient TS; κ gene rearrangements are not detectable in *Eco*RI digests with a C_κ probe (6). PMT-3 is a plasmid marker lane, as in Fig. 1; fragment sizes of the PMT-3 bands and some of the J_H bands are indicated to the left and right of the radiograms.

often become oligoclonal or monoclonal with time has been reported, including recent evidence gathered by the analysis of immunoglobulin gene rearrangements (3-5, 9, 13). The application of molecular genetic analyses to the clonally derived, EBV-transformed B-cell lineages from our lymphoma patient resulted in documentation, for the first time, of a metastatic cell lineage arising in the context of a fatal, EBV-induced B lymphoproliferation, a feature which could not be established by the experimental methods utilized for previous reports. Thus, our data provide further support for the idea that detailed studies of the lymphoproliferative process in immunodeficient persons may allow unique insights regarding benign versus malignant progressions of EBV-induced B lymphoproliferations.

We thank Leroy Hood (California Institute of Technology) and Philip Leder (Harvard University), and their colleagues, for donation of the immunoglobulin gene probes used in this work. Helga Muller prepared the Giemsa-banded chromosomes for cell clone TS-S4. Rick Ponce assisted with some of the DNA preparations. Leslie Spring prepared the manuscript.

This work was supported by Public Health Service grant CA35536 from the National Cancer Institute, grant G821118 from the California Institute for Cancer Research, a University of California at Los Angeles Faculty Senate Research award, and Public Health Service Biomedical Research Support grant RR05354 from the National Institutes of Health.

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