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Epstein-Barr Virus Induces Fragmentation of Chromosomal DNA during Lytic Infection

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Pulsed-field agarose gel electrophoresis showed that fragmentation of chromosomal DNA in Raji cells was induced by infection with the P3HR-1 strain of Epstein-Barr virus (EBV). S1 nuclease treatment of the agarose plugs containing cells suggested that the majority of DNA fragments did not contain single-strand gaps. Chromosomal DNA fragmentation was inhibited by cycloheximide, indicating that protein synthesis was required for DNA fragmentation. Phosphonoacetic acid, an inhibitor of EBV DNA polymerase, did not inhibit fragmentation of chromosomal DNA. These findings suggest that EBV-specific early proteins participate in fragmentation of chromosomal DNA. Chromosomal DNA of P3HR-1 cells was also fragmented by treatment with *n*-butyrate plus 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which induced activation of latent EBV genome following viral replication. In addition, fragmentation of DNA preceded cell death during lytic infection. These results suggest that fragmentation of chromosomal DNA is generally induced during EBV replication and probably contributes to the cytopathic effect of EBV. The role of DNA fragmentation in death of infected cells is discussed in relation to apoptosis.

Herpesviruses, in general, infect cells in two different ways: (i) productive infection with replication of progeny viruses followed by death of the affected cells and (ii) nonproductive, noncytocidal infection (6). Epstein-Barr virus (EBV), a human herpesvirus, is the causative agent of infectious mononucleosis and is closely associated with Burkitt's lymphoma and nasopharyngeal carcinoma (9). EBV infects human B lymphocytes and transforms infected cells (12). In transformed B cells, the virus is usually latent and a small number of particular viral genes are expressed (including EBV nuclear antigens 1 to 4, the leader protein, latent membrane proteins, and two small RNAs called EBER1 and EBER2) (4), but in some transformed B-cell lines, a low percentage of cells spontaneously produce progeny viruses. However, viral productive cycle can be induced in a high percentage of cells by superinfection with EBV, by treatment with chemical inducers such as *n*-butyrate or 12-*O*-tetradecanoylphorbol-13-acetate (TPA), or by antibody to human immunoglobulin M (10, 17, 23, 27). Once cells enter the viral productive cycle, as many as 80 EBV genes are expressed and the host cell is eventually killed (2, 11, 13, 21).

The mechanism of death of host cells infected with EBV remains to be clarified. Recently, the association of viral cytotoxic response with apoptosis has been demonstrated for several viruses. In addition, viral transcripts which induced apoptosis were identified. Adenovirus E1A proteins induce apoptosis, which is inhibited by E1B 19-kDa and Bcl-2 oncogene proteins (20, 24, 25). In acute human immunodeficiency virus (HIV) infection, low-molecular-weight DNA in the form of nucleosomes accumulates in the nucleoplasm of infected cells, demonstrating that the cytopathic effect of HIV is associated with apoptosis (15, 22). Apoptosis is an active form of physiological cell death which differs morphologically and biochemically from necrosis (5, 12). Features that distinguish apoptosis from necrosis are induction of chromatin condensation and nuclear DNA fragmentation in intranucleosomal regions (1, 26). The purpose of the present study was to clarify the mechanisms of cytocidal action of EBV during lytic infection in relation to fragmentation of host cell DNA. Chromosomal DNA in EBV-infected cells was analyzed by pulsed-field gel electrophoresis (3).

Raji cell line and two P3HR-1 sublines lacking or containing heterogenous EBV DNA (htDNA) were used. Cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (250 μg/ml). EBV was obtained by concentrating the culture fluid of the P3HR-1 subline containing htDNA 100 times. After adsorption with concentrated virus at the indicated multiplicity of infection at 37°C for 1 h, Raji cells were suspended in fresh medium and incubated at 37°C. The multiplicity of infection was calculated by slot blot hybridization of EBV-specific DNA from mock-infected and infected Raji cells 2 h postinfection, using a laser densitometer (LKB 2222 UltroScan XL). P3HR-1 cells lacking htDNA were treated with 5 mM *n*-butyrate plus 20 ng of TPA per ml.

For pulsed-field gel analysis, cells were washed with cold phosphate-buffered saline (PBS) three times and resuspended in PBS at the concentration of 2×10^6 cells per ml. The cell suspension was mixed with an equal volume of 1.3% low-melting-point agarose, poured into sample holders. The agarose plugs were transferred in 1-ml portions of NDS buffer (0.5 M EDTA, 0.01 M Tris, 1% *n*-lauryl sarcosine, 1 mg of proteinase K per ml) and incubated at 50°C for 48 h. Electrophoresis was performed in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1.25 mM EDTA; pH 8.3) by pulsed-field (CHEF-DRII DNA megabase electrophoresis system; Bio-Rad) at 200 V at 14°C. Switch time was 60 s for 15 h, followed by 90-s switch time for 9 h. The DNA in the gel was visualized by staining with 0.5 μg of ethidium bromide per ml. *Saccharomyces cerevisiae* chromosomes (Bio-Rad) were used as DNA size markers. For Southern blot analysis, gels were deprinated with 0.25 HCl, denatured with 0.4 N NaOH and 1.5 M NaCl, and neutralized. DNA was transferred into nitrocellulose filters. Filters were baked at 80°C for 2 h, hybridized with the *Bam*HI W EBV DNA fragment labelled with [^{32}P]dCTP by nick translation, and exposed to RP X-Omat film (Kodak) with an intensifying screen.

For analysis of nucleosomal DNA fragments, cells were disrupted in a solution containing 10 mM Tris-HCl (pH 7.6), 0.15 M NaCl, 5 mM $MgCl_2$, and 0.5% Triton X-100. After centrifugation at 1,000 × *g*, the intact nuclei were pelleted.

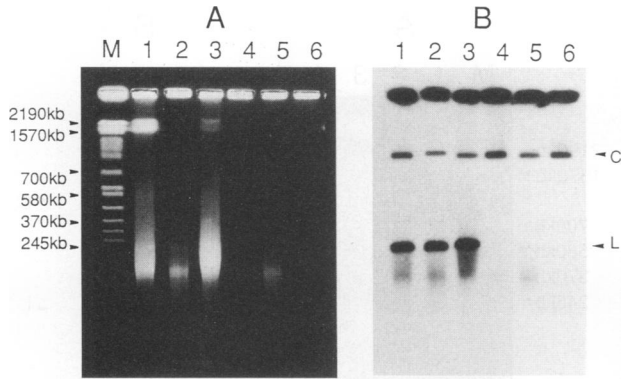

FIG. 1. Pulsed-field gel analysis of DNA from Raji cells infected with P3HR-1 virus. Infected (lanes 1 to 3) and mock-infected (lanes 4 to 6) Raji cells were harvested at 24 h postinfection and analyzed by pulsed-field gel electrophoresis. DNA was transferred onto nitrocellulose filters and hybridized with ^{32}P-labelled *Bam*HI W EBV DNA fragment. (A) Gel stained with ethidium bromide. (B) Southern blot analysis of EBV DNA. Lanes 1 and 4, untreated cells; lanes 2 and 5, cells treated with cycloheximide (25 μg/ml); lanes 3 and 6, cells treated with PAA (300 μg/ml). Lane M, yeast chromosomes used as DNA size markers. C, circular form of EBV DNA; L, linear form of EBV DNA.

The nuclei were then extracted in a solution containing 10 mM Tris-HCl (pH 7.6), 0.4 M NaCl, 1 mM EDTA, and 1% Triton X-100 and centrifuged at 12,000 × *g* to separate the nucleoplasm from the high-molecular-weight chromatin. Nucleoplasm was incubated with 20 μg of RNase per ml for 1 h at room temperature and then with 100 μg of proteinase K per ml for 2 h at 37°C. DNA was purified with phenol and chloroform and analyzed by conventional electrophoresis on 1.4% agarose gels containing 0.5 μg ethidium bromide per ml at 60 V for 3 h.

It is well-known that EBV productive cycles are induced in Raji cells by superinfection with P3HR-1 virus and that infected cells eventually die. The time course of cell killing by EBV infection was confirmed in the present cell system (data not shown). Raji cells were infected with P3HR-1 virus at a multiplicity of infection of 30 in the presence or absence of phosphonoacetic acid (PAA), a specific inhibitor of EBV DNA polymerase. Cells were stained with trypan blue at the indicated time. At 24 h postinfection, more than 95% of infected cells were viable in the presence or absence of PAA, and more than 90% of infected cells in the absence of PAA were positively stained by indirect immunofluorescence with serum from a patient with nasopharyngeal carcinoma and containing antibodies to early antigen and viral capsid antigen. However, after 48 h viable cells decreased in number with time postinfection. At 72 h postinfection, approximately 50% and more than 85% of cells died in the absence and presence of PAA, respectively. These results show that Raji cells infected with EBV are eventually killed and that the cytocidal action of EBV is enhanced in the presence of PAA.

Chromosomal DNA in Raji cells infected with P3HR-1 EBV at 24 h postinfection was analyzed by pulsed-field gel electrophoresis followed by ethidium bromide staining. As shown in Fig. 1A, in mock-infected Raji cells, intact chromosomal DNA was detected at the top of the gel and no apparent fragmented DNA were detected (lane 4). In contrast, in infected Raji cells, DNA fragments of 2,100 and 1,400 kbp and continuous smear DNA with molecular size less than 700 kbp appeared in addition to intact chromosomal DNA (Fig. 1A, lane 1). To determine whether the fragmented DNA was derived from chromosomal DNA or amplified EBV DNA, the effect of PAA on DNA fragmentation was examined. Raji cells infected with EBV in the presence of PAA were harvested at 24 h postinfection and analyzed by pulsed-field gel electrophoresis. As shown in Fig. 1A, lane 3, DNA fragmentation was not blocked by PAA. Therefore, the bulk of fragmented DNA visualized by staining with ethidium bromide in the infected cells must be fragmented host cell DNA, because viral DNA replication is inhibited by PAA. DNA fragmentation of infected cells in the presence of PAA was not due to the direct effect of PAA, because no apparent DNA fragmentation was detected in the mock-infected cells treated with PAA (Fig. 1A, lane 6).

The results obtained from the above experiments were further confirmed by Southern blot hybridization of the pulsed-field gel. DNA was transferred onto nitrocellulose filters and hybridized with ^{32}P-labelled *Bam*HI W EBV DNA fragment. In mock-infected Raji cells, only circular viral genome was detected (Fig. 1B, lane 4). In infected Raji cells, 175-kb linear EBV genomic DNA was detected in addition to circular EBV DNA (Fig. 1B, lane 1). The linear EBV DNA was considered to be exogenous and progeny EBV DNA. A similar pattern of EBV DNA was observed in infected Raji cells in the presence of PAA (Fig. 1B, lane 3). Distribution of DNA on the gel visualized by ethidium bromide staining was clearly different from that of EBV DNA identified by Southern blot hybridization. On the basis of these findings, it was concluded that 2,100- and 1,400-kbp DNA and smear DNA with lower molecular weight visualized by ethidium bromide were cleaved from chromosomal DNA by EBV infection.

To clarify whether the DNA of 2,100 and 1,400 kbp was an artifact of single-strand gaps in the DNA, the effect of S1 nuclease was tested. If the DNA was an artifact, the DNA with molecular size less than 700 kbp should be increased by treatment with S1 nuclease. The agarose plugs were incubated in LET buffer (0.5 M EDTA, 0.01 M Tris [pH 7.5], 7.5% 2-mercaptoethanol), which was used for enhancement of cell lysis and deproteinization, at 37°C for 24 h and then treated with NDS buffer at 50°C for 24 h. After pretreatment with 1 mM PMSF (phenylmethylsulfonyl fluoride), the deproteinized agarose plugs were digested with S1 nuclease (5 U) at 37°C for 30 min. As shown in Fig. 2, in infected Raji cells, 2,100- and 1,400-kbp DNA bands increased in intensity by treatment with S1 nuclease, but the patterns of continuous smear DNA with molecular size less than 700 kbp were quite similar before and after treatment with S1 nuclease (lanes 4 and 5). Therefore, it was concluded that the majority, if not all, of 2,100- and 1,400-kbp DNA fragments were not artifacts but double-stranded DNA produced by EBV infection. Increase in the intensity of 2,100- and 1,400-kbp DNA bands after treatment with S1 nuclease is probably due to S1 nuclease digestion of intact chromosomal DNA containing single-stand gaps, because the intensities of these two DNA bands also increased in mock-infected cells by S1 digestion (Fig. 2, lanes 2 and 3). It is well-known that single-strand breaks occur spontaneously and also during DNA replication.

When protein synthesis was blocked by the addition of cycloheximide during infection, fragmentation of chromosomal DNA was completely inhibited (Fig. 1A, lane 2). The formation of a small amount of low-molecular-weight DNA in infected Raji cells in the presence of cycloheximide was considered to be due to the direct effect of cycloheximide, because the same level of DNA fragmentation was induced in the mock-infected cells by treatment with cycloheximide (Fig. 1A, lane 5). DNA fragmentation increased with the time postinfection, and the time course correlated with those of

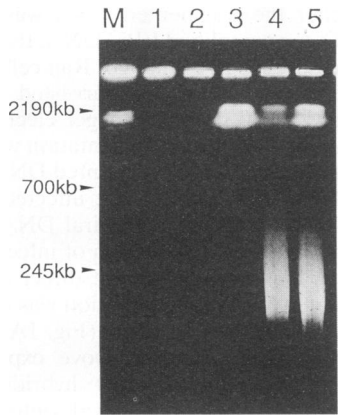

FIG. 2. Effect of S1 nuclease on fragmented DNA. Mock-infected (lanes 1 to 3) and infected (lanes 4 and 5) Raji cells were harvested at 24 h postinfection. After deproteinization by NDS buffer following treatment with LET buffer, the agarose plugs were incubated in S1 buffer without (lanes 2 and 4) or with S1 nuclease (lanes 3 and 5) at 37°C for 30 min and analyzed by pulsed-field gel electrophoresis. Lane 1, deproteinized agarose plug; lane M, yeast chromosomes used as DNA size markers.

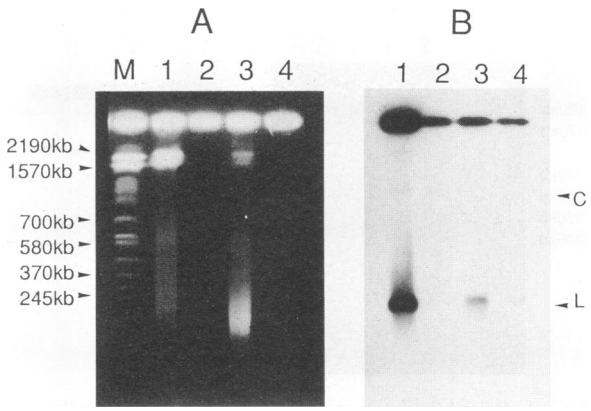

FIG. 3. Pulsed-field gel analysis of DNA from chemically treated P3HR-1 cells. P3HR-1 cells untreated (lanes 4) or treated with TPA plus *n*-butyrate (lanes 1 to 3) were analyzed by pulsed-field gel electrophoresis as described in the legend to Fig. 1. (A) Gel stained with ethidium bromide. (B) Southern blot analysis of EBV DNA. Lanes 1, untreated cells; lanes 2, treated cells in the presence of cycloheximide (25 μg/ml); lanes 3, treated cells in the presence of PAA (300 μg/ml); lane M, yeast chromosomes used as DNA size markers. C, circular form of EBV DNA; L, linear form of EBV DNA.

syntheses of EBV-specific proteins identified by immunoblotting (data not shown). Furthermore, fragmentation of chromosomal DNA was increased by increasing the multiplicity of infection, and the density of each EBV-specific polypeptide identified by immunoblot technique increased with the multiplicity of infection (data not shown). These results together suggest that EBV-specific proteins or cellular proteins induced by EBV infection are involved in chromosomal DNA damage. Because PAA represses the synthesis of late proteins of EBV, fragmentation of chromosomal DNA must be due to proteins produced early in infection, although which molecule among EBV transcripts is associated with chromosomal DNA fragmentation and by what mechanism chromosomal DNA is fragmented remain to be elucidated.

Because only a small amount of progeny viral DNA was replicated in infected Raji cells under the present conditions, chemically treated P3HR-1 cells were examined for DNA fragmentation. P3HR-1 cells were treated with TPA and *n*-butyrate for 24 h and analyzed by pulsed-field gel electrophoresis followed by ethidium bromide staining. DNA was transferred onto nitrocellulose filters and hybridized with ^{32}P-labelled *Bam*HI W EBV DNA fragment. At 24 h after treatment, cell viability was more than 90%, and 80 to 85% of cells were positive for early antigen or viral capsid antigen. In addition, numerous amplified EBV DNAs were detectable (Fig. 3B, lane 1). In these cells, a fragmentation pattern of chromosomal DNA quite similar to that in infected Raji cells was detected (Fig. 3A). These results suggest that fragmentation of chromosomal DNA seems to be a general phenomenon during lytic cycle of EBV. Fragmentation of chromosomal DNA in chemically treated P3HR-1 cells was also inhibited by cycloheximide (Fig. 3A, lane 2).

When DNA patterns of superinfected Raji (Fig. 1A, lanes 1 and 3) or chemically activated P3HR-1 (Fig. 3A, lanes 1 and 3) cells in the absence and presence of PAA were compared, DNA fragmentation was more pronounced by treatment with PAA. The reason why DNA fragmentation is enhanced in the presence of PAA is not yet clear. It may be related to the overproduction of one or more EBV early proteins in the absence of DNA replication and late transcripts. However, EBV-specific proteins overproduced in the presence of PAA were not identified by immunoblot analysis (data not shown) or by the immunoprecipitation analysis previously demonstrated (11), using sera from patients with nasopharyngeal carcinoma. The discrepancy may be explained by the sensitivities of these methods or by deficiency of antibodies against such proteins in the sera used. Enhanced fragmentation of chromosomal DNA by PAA probably results in a more severe cytocidal response in the presence of PAA than that in the absence of PAA (data not shown).

The next experiment was designed to determine whether chromosomal DNA fragmentation detected by pulsed-field gel electrophoresis leads to oligonucleosome-length DNA characteristic of apoptosis. Low-molecular-weight DNA was analyzed by conventional 1.4% agarose gel electrophoresis, because pulsed-field gel electrophoresis resolved high-molecular-weight DNA molecules from 200 to 2,000 kbp, but not oligonucleosome-length DNA fragments. Nucleosome laddering characteristic of apoptosis was observed in Jurkat cells cultivated in a low concentration of fetal calf serum (Fig. 4, lane 5). However, such fragmentation patterns were not detectable in Raji cells, even at 72 h postinfection in the presence (Fig. 4, lane 4) or absence (Fig. 4, lane 3) of PAA. In addition to analysis of the DNA laddering, acridine orange staining clearly distinguishes between viable cells and cells showing the condensed chromatin staining characteristic of apoptosis (18). However, cells with chromatin condensation were rarely detected among the infected cells even at 72 h postinfection (data not shown) when more than 50% of cells were already dead.

The present study shows that fragmentation of chromosomal DNA in Raji cells is induced by superinfection with P3HR-1 strain of EBV using pulsed-field gel electrophoresis. This result is consist with previous studies which demonstrated breakdown of cellular DNA in EBV infection by alkali sucrose gradient centrifugation (19). However, it is not possible to use alkali sucrose centrifugation to characterize fragmented DNA. In contrast, pulsed-field gel electrophoresis permits the separation of intact chromosomal DNA from degraded chromosomal DNA or viral DNA without any artifacts and the

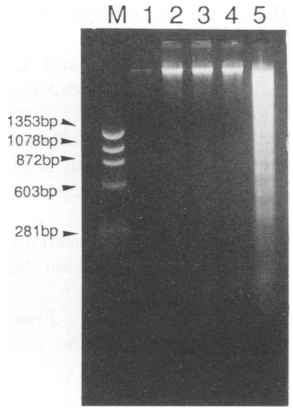

FIG. 4. Analysis of total cellular DNA by conventional agarose gel electrophoresis. Total cellular DNA was extracted from mock-infected cells (lane 1), infected cells harvested at 48 (lane 2) or 72 (lane 3) h postinfection in the absence of PAA, or infected cells treated with PAA for 72 h (lane 4). DNA was analyzed by 1.4% agarose gel electrophoresis. Lane 5 shows the gel electrophoresis pattern of DNA from Jurkat T-cell line cultivated in medium supplemented with 0.1% fetal calf serum for 4 days. Lane M contains *Hae*III-digested ϕX174 DNA as DNA size markers.

characterization of fragmented DNA in the native state. It is reasonable to consider that once chromosomal DNA has been fragmented, the cells are going to die. In general, cytotoxicity correlated with drug-induced DNA cleavage (16). Therefore, it seems likely that chromosomal DNA damage induced by superinfection plays a critical role in cell death caused during EBV lytic infection, although the entire mechanism of cell death during EBV lytic infection remains to be elucidated.

The findings presented here are noteworthy in relation to apoptosis. DNA fragmentation characteristic of apoptosis requires protein synthesis and precedes the first signs of cell death, whereas during necrosis the DNA is fragmented into a continuous spectrum of sizes as a result of simultaneous action of both lysosomal proteases and nucleases, released in cells already dead (5, 14). On the basis of these criteria, the process of DNA fragmentation detected in the lytic cycle of EBV resembles that characteristic of apoptosis and is apparently distinguished from DNA fragmentation during necrosis for the following reasons. Fragmented chromosomal DNA in EBV-infected cells appeared as early as 8 h postinfection, and DNA fragmentation increased with the time postinfection. In addition, the findings that more than 95% of the infected cells remain viable (exclude trypan blue) at 24 h postinfection and dead cells increase in number after 48 h postinfection indicate that chromosomal DNA damage precedes cell death. Furthermore, fragmentation of host cell DNA was inhibited by cycloheximide, indicating that protein synthesis was required for DNA fragmentation induced by EBV infection. However, in the EBV-infected cells, typical laddered DNA fragmentation patterns characteristic of apoptosis were not detected at 48 h and even at 72 h postinfection, when approximately 50% of the cells were already dead. It may be considered that EBV infection triggers apoptosis, resulting in fragmentation of chromosomal DNA detected by pulsed-field gel electrophoresis, but the resultant fragmented DNA is prevented from cleavage into nucleosome-length DNA characteristic of apoptosis by unknown mechanisms. Cell death induced by EBV infection might be associated not with typical apoptosis but with "quasi-apoptosis," when intermediate phase of DNA fragmentation into nucleosome-length DNA could be detected. EBV latent membrane protein 1 (LMP-1) might be a candidate which interferes with DNA fragmentation of host DNA into characteristic nucleosome ladders, because LMP-1 has been shown to inhibit apoptosis (8). In fact, LMP-1 is expressed in both Raji and P3HR-1 cells (7).

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