Reactivation of Latent Epstein–Barr Virus by Methotrexate: A Potential Contributor to Methotrexate-Associated Lymphomas

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Background: Patients with rheumatoid arthritis or polymyositis treated with methotrexate (MTX) develop Epstein–Barr virus (EBV)–positive lymphomas more frequently than patients treated with other, equally immunosuppressive regimens. Here we determined whether MTX, in contrast to other commonly used medications for rheumatoid arthritis or polymyositis, is unique in its ability to induce the release of infectious EBV from latently infected cells.

Methods: The effect of MTX and other immunosuppressant drugs on EBV replication in vitro was assessed using latently infected EBV-positive lymphoblastoid and gastric carcinoma cell lines. Inhibitors of signal transduction pathways were used to define requirements for induction of lytic infection. Drug effects on transcription of the two EBV immediate-early promoters (BRLF1 and BZLF1) and on promoter constructs lacking cis-acting sequences required for activation by other effectors was examined using reporter gene assays. EBV viral load in rheumatoid arthritis and polymyositis patients receiving MTX was compared with that in patients receiving other immunosuppressive medications. Statistical tests were two-sided. Results: MTX activated the release of infectious EBV from latently infected cell lines in vitro, and MTX treatment was associated with activation of the two viral immediate-early promoters in reporter gene assays. Induction of lytic EBV infection by MTX required the p38 MAP kinase, PI3 kinase, and MEK pathways and specific cis-acting motifs in the two viral immediate-early promoters. Patients treated with MTX-containing regimens had statistically significantly higher mean EBV loads in their blood than patients treated with immunosuppressing regimens that did not include MTX (40 EBV copies per 10^6 cellular genomes versus 5.1 copies; geometric mean fold difference in copies = 10.8, 95% confidence interval = 3.0 to 38; P = .011). Conclusion: MTX may promote EBV-positive lymphomas in rheumatoid arthritis and polymyositis patients by its immunosuppressive properties as well as by reactivating latent EBV. [J Natl Cancer Inst 2004;96:1691–1702]

Epstein–Barr virus (EBV) is a ubiquitous human herpesvirus that establishes a lifelong persistent infection of B cells in more than 90% of the human adult population. EBV is a B-lymphotropic virus that is characterized by its ability to transform B cells in vitro, and it is associated with a variety of B-cell disorders, including infectious mononucleosis, African Burkitt

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lymphoma, Hodgkin lymphoma, and, in immunosuppressed individuals, lymphoproliferative disorders (1–3). Patients receiving the immunosuppressant drug methotrexate (MTX) for rheumatoid arthritis or for polymyositis are at increased risk, compared with the general population, of developing EBV-associated lymphoproliferative disorders (4–7).

Like other herpesviruses, EBV can infect cells in either a latent or a lytic manner (1,2). Most EBV-infected B cells are latently infected. In such cells, only a small number of the total viral genes are expressed, viral DNA is replicated using the host cell DNA polymerase, and the host cell is not killed. The EBV proteins associated with cellular transformation (in particular, LMP1) are expressed in certain forms of latent infection, and most of the tumor cells in EBV-associated lymphoproliferative disorders express transformation-associated viral proteins.

The switch from latent to lytic EBV infection is mediated by expression of the two EBV immediate-early (IE) viral proteins, BZLF1 and BRLF1. BZLF1 and BRLF1 encode transcriptional activators that, in combination, activate the complete cascade of lytic viral gene expression. In the lytic form of viral infection, many additional viral gene products are transcribed, the virus is replicated using a virally encoded DNA polymerase, infectious viral particles are released, and the host cell is killed (1,2).

Although cellular transformation results from the latent, rather than lytic, form of EBV infection, an increased level of lytically infected cells in patients may nevertheless increase the likelihood of an EBV-associated malignancy. A persistently high level of circulating infectious EBV particles would be predicted to increase the number of latently infected B cells, from which the pool of potentially transformed cells arises. Indeed, AIDS patients and organ transplant patients, who have a high risk of developing EBV-associated lymphomas, have a high level of lytic as well as latent EBV infection (8–12). Although it is controversial, there is some evidence to suggest that prophylactic treatment of organ transplant patients with antiviral drugs that prevent the lytic form of EBV replication may reduce the incidence of EBV-associated lymphomas in these patients (13–16).

Patients with rheumatoid arthritis or polymyositis have an increased susceptibility to B-cell lymphomas compared with the general population (17,18). Interestingly, a number of reports have suggested that the frequency of EBV-positive lymphomas, including Hodgkin lymphoma, is increased in rheumatoid arthritis and polymyositis patients treated with MTX (5,6,19–21). Moreover, MTX-associated lymphomas in some rheumatoid arthritis patients have been reported to regress, at least partially, following MTX withdrawal (4,5,20,22–24). The mechanisms by which MTX increases the propensity for rheumatoid arthritis and polymyositis patients to develop EBV-positive lymphomas are not entirely clear. The immunosuppressive effect of MTX is probably an important component, because immunosuppression is known to predispose patients to EBV-related lymphomas. Nevertheless, MTX-induced immunosuppression by itself is unlikely to be a sufficient explanation for the development of EBV-positive lymphomas in rheumatoid arthritis and polymyositis patients because equally immunosuppressive drugs have not been associated with EBV-positive lymphomas in these patients. In addition, patients with the inflammatory disorder Wegener granulomatosis treated with MTX have not been reported to have an increased propensity for EBV-associated lymphoproliferative disorders.

We recently found that certain chemotherapy agents, including gemcitabine, doxorubicin, cis-platinum, and 5-fluorouracil, induce lytic EBV gene transcription in latently infected EBV-positive cell lines (25,26). We therefore hypothesized that MTX may similarly induce lytic EBV infection in host cells, resulting in the release of infectious EBV virions. In this study, we tested our hypothesis in vitro by analyzing lytic EBV gene expression in and the release of infectious virions from EBV-positive lymphoblastoid cell lines and AGS gastric carcinoma cells treated with MTX and other chemotherapy drugs. We have previously shown that some inducing agents (e.g., cis-platinum and 5-fluorouracil) efficiently activate the lytic form of EBV infection in AGS gastric carcinoma cells but are ineffective in this regard in lymphoblastoid cell lines (25,26). We also analyzed the activation of the two EBV IE promoters in MTX-treated cells and used inhibitors to investigate the involvement of the cellular p38 MAP kinase, PI3 kinase, and MAP kinase kinase (MEK) signal transduction pathways. Finally, we examined EBV loads in patients with rheumatoid arthritis and polymyositis treated with MTX and with other immunosuppressive drugs.

MATERIALS AND METHODS

Cell Lines

Three latently EBV-infected lymphoblastoid cell lines (LCLs) were obtained by transforming primary human B cells from three different donors with the B95-8 strain of EBV. Raji cells, an EBV-positive Burkitt lymphoma cell line, were obtained from American Type Culture Collection (Manassas, VA). DG75 cells, an EBV-negative Burkitt lymphoma cell line, were obtained from G. Klein (Karolinska Institute, Stockholm, Sweden) and were used in the chloramphenicol acetyltransferase (CAT) assays. AGS, a gastric carcinoma cell line, was obtained from American Type Culture Collection. The AGS-EBV-GFP cell line was created by infecting AGS cells with a modified B95-8 strain of EBV, which contains an inserted green fluorescence protein (GFP) gene. The GFP-containing EBV was constructed using bacterial artificial chromosome technology, as previously described (27). Cells were cultured in RPMI-1640 medium (all suspension cells) or Ham’s F-12 medium (the AGS-EBV-GFP cell line) with 10% fetal bovine serum (FBS) at 37 °C with 5% CO₂ and 100% humidity.

Lytic Induction Assays

Lytic induction assays were performed using either LCLs that had been grown to a density of 5 × 10⁵ cells/mL in RPMI medium with 10% FBS or AGS-EBV-GFP cells that were plated at approximately 8 × 10⁶ cells in 100-mm plates and grown for 2 days in Ham’s F-12 medium with 10% FBS. Cells were treated with the following immunosuppressants: MTX ([Immunex, Thousand Oaks, CA] 0.1, 0.5, 5, or 50 μg/mL suspended in isotonic sodium chloride), azathioprine ([Bedford Laboratories, Bedford, OH] 1 or 10 μg/mL diluted in sterile water), cyclosporine ([Novartis, Basel, Switzerland] 1 or 10 μg/mL diluted in isotonic sodium chloride), cyclophosphamide ([MeadJohnson, Princeton, NJ] 10 or 100 μg/mL diluted in sterile water), mycophenolic acid ([Sigma, St. Louis, MO] 10 or 50 μg/mL suspended in dimethyl sulfoxide [DMSO]), or prednisone ([Sigma] 1 or 10 μM suspended in DMSO) for 48 hours (for AGS-EBV-GFP cells) or 72 hours (for LCLs). By comparison,
the predicted peak serum levels of the drugs used were as follows: methotrexate given at 15 mg orally each week in an adult, 0.4 μg/mL; azathioprine given at 2 mg/kg/day orally, up to 1 μg/mL; mycophenolate given at 1 g twice a day orally, 20-25 μg/mL (mycophenolic acid metabolite); prednisone given at 20 mg/day orally, 55–85 nM; and cyclosporin (Neoral) given at 2.5 mg/kg/day orally, 400–800 ng/mL. Cyclophosphamide is metabolized extensively into multiple active and inactive metabolites in vivo and thus peak serum levels are difficult to predict. The methotrexate doses spanned a wide range and included doses achievable in rheumatoid arthritis patients during prolonged treatment courses. Experiments using the other immunosuppressive drugs included doses that were at least as high as the peak serum levels attainable in patients. Gemcitabine (Eli Lilly, Indianapolis, IN) 1 μg/mL diluted in isotonic sodium chloride) and doxorubicin (Ben Venue Laboratories, Bedford, OH) 0.2 μM diluted in isotonic saline) were also used in some experiments. Following drug treatment, whole cell extracts were obtained by lysing cells in lysis buffer (0.25 M NaCl, 0.1% NP-40, 0.05 M HEPES [pH 7.0], 0.001 M phenylmethylsulfonyl fluoride, 0.005 M EDTA, 0.5 mM dithiothreitol). Immunoblot analysis of the IE lytic proteins BRLF1 and BZLF1 and the early lytic protein BMRF1 was performed as described previously (28) using the following primary monoclonal antibodies: anti-BMRF1 (1:100; Capricorn, Portland, ME), anti-BZLF1 (1:100; Argene, North Massapequa, NY), anti-BRLF1 (1:100; Argene), and β-actin (1:5000; Sigma). Briefly, 30 μg of protein for each sample was loaded in each lane and separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The proteins were transferred overnight onto a nitrocellulose membrane (Osmonics, Westborough, MA), blocked in 1x phosphate-buffered saline–5% milk–0.1% Tween 20, and incubated in primary antibody for 1 hour at room temperature. The membrane was washed in phosphate-buffered saline–0.1% Tween 20, incubated in secondary antibody (anti-mouse immunoglobulin G–horseradish peroxidase conjugate, 1:10 000 [Promega, Madison, WI]) for 1 hour at room temperature, and washed again, and the results were visualized with the enhanced chemiluminescence detection kit (GE Health Care, Piscataway, NJ).

Inhibition of Signal Transduction Pathways

LCLs, grown to a density of 5 × 10⁵ cells/mL in RPMI-1640 medium with 10% FBS, were pretreated for 1 hour with either vehicle alone (DMSO), the PI3 kinase inhibitor LY294002 (15 μM in DMSO; Calbiochem, La Jolla, CA), the p38 MAPK inhibitor SB202190 (20 μM in DMSO; Calbiochem), the MEK inhibitor PD98059 (50 μM in DMSO; Calbiochem), or the pan-caspase inhibitor Z-VAD-fmk (50 μM in DMSO; Calbiochem). Cells were then treated for 72 hours with or without MTX (50 μg/mL) in the presence or absence of the inhibitors and harvested for immunoblot analysis for BMRF1, BZLF1, BRLF1, LMP1 and β-actin expression. Experiments were performed twice.

EBV Promoter Plasmids

Plasmid RpCAT contains the BRLF1 IE promoter (Rp) sequences (from −962 to +5 relative to the mRNA start site) linked to a heterologous reporter gene, CAT, in the pBS phage-mid vector (Stratagene, La Jolla, CA) (29). RpCAT (∆Zif) contains Rp sequences (from −962 to +5) with two site-directed mutations that delete the upstream (from −131 to −125) and downstream (from −44 to −42) Zif268 (EGR-1) binding sites (30). Plasmid ZpCAT contains the BZLF1 IE promoter (Zp) (from −221 to +12) linked to CAT (31). Plasmid ZpCAT (∆ZIA/B) contains Zp sequences with two of the ZI motifs, ZIA and ZIB (both of which are binding sites for MEF2D) (32), mutated as previously described (31). ZpCAT (ZII) contains Zp sequences with a site-directed mutation in the ZII (CRE) binding site (31). All plasmids were grown in DH5-α Escherichia coli bacteria and purified using a plasmid DNA isolation kit (Qiagen, Valencia, CA), as described by the manufacturer.

CAT Assays

Plasmid DNA (5 μg) was transfected into EBV-negative DG75 cells (2 × 10⁷ cells in 0.5 mL of medium) by electroporation at 1500 V with a Zapper electroporation unit (Medical Electronics Shop, University of Wisconsin). Immediately after transfection, cells were resuspended in 5 mL of RPMI-1640 medium with 10% FBS. After 12 hours, cells were treated with or without 50 μg/mL MTX for 72 hours. Cell extracts were prepared by pelleting the transfected cells in a microcentrifuge (Heraeus Biofuge; Sorvall, Newtown, CT) at 15 000 g for 5 minutes and then resuspending the pellet in 200 μL of 0.25 M Tris (pH 7.5). One hundred microliters of the extract was incubated at 37 °C with 0.25 μL of [14C]chloramphenicol (0.2 μCi/μL; GE Health Care) in the presence of acetyl coenzyme A, as previously described (33). The percent acetylation of chloramphenicol was quantified by thin-layer chromatography followed by Phosphorimager screening (Molecular Dynamics, GE Health Care). The CAT activity of each construct in cells without methotrexate treatment was normalized to 100%.

EBV DNA Terminus Assay

To evaluate the relative levels of the episomal and linear structures of the EBV genome, which represent latent and lytic infection, respectively, AGS-EBV-GFP cells (at a density of approximately 8 × 10⁶ cells/100-mm plate in Ham’s F-12 medium with 10% FBS) were treated for 48 hours with the following conditions: a) no drug, b) 75 μM acyclovir (Siga-Aldrich, St. Louis, MO; [antiviral drug] diluted in sterile water), c) 1 μg/mL gemcitabine, d) 50 μg/mL MTX, e) gemcitabine and acyclovir combined, or f) MTX and acyclovir combined. Cells were harvested by centrifugation in a microcentrifuge at 13 000 rpm for 5 minutes. DNA was isolated using DNeasy Tissue Kit (Qiagen) following the DNeasy Protocol for cultured animal bacteria and purified using a plasmid DNA isolation kit (Qiagen, Valencia, CA), as described by the manufacturer.

Detection of Infectious EBV

AGS-EBV-GFP cells were plated at approximately 8 × 10⁶ cells in 100-mm plates and grown in Ham’s F-12 medium with 10% FBS for 48 hours with the following treatments: a) no drug, b) 75 μM acyclovir, c) 1 μg/mL gemcitabine, d) 50 μg/mL MTX, e) gemcitabine and acyclovir combined, or f) MTX and acyclovir combined. The medium was then replaced with medium lacking drugs, and 3 days later, supernatants (containing infectious EBV particles) from the AGS-EBV-GFP cells were collected by centrifuging 10 mL of the medium at 200g for 5
minutes in an IEC HN-SII centrifuge and filtering the supernatant through a 0.45-μm filter. Extracts of the AGS-EBV-GFP cells were also collected for BMRF1 immunoblot analysis as described above. The amount of infectious EBV in the AGS-EBV-GFP supernatants was determined by superinfecting 1 × 10^6 Raji cells in 1 mL of RPMI medium with 10% FBS) with 1 mL of each supernatant and, 3 days later, quantitating the level of GFP expression in the Raji cells by immunoblot analyses, as described above, using a primary antibody directed against GFP (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) or β-actin.

**Patient Samples and DNA Extraction**

Blood was obtained from 29 consecutive patients with rheumatoid arthritis, 12 consecutive patients with polymyositis, and 47 consecutive patients with Wegener granulomatosis who were on Internal Review Board–approved clinical research protocols at the Warren Grant Magnuson Clinical Center at the National Institutes of Health in Bethesda, MD. Protocols for studying these patients include analysis of laboratory tests, such as EBV DNA amplification and quantification

EBV DNA was quantified in patient PBMCs using the LightCycler system (Roche Molecular Biochemicals, Indianapolis, IN). In brief, primers EBV FOR.4 (5'-AGGAAGCCGGTTCTAT GTGTGCGTCG-3') and EBV REV.5 (5'-TAGAAGCTGACAAA TTGCGCTGCTGCTGCTG-3'), where S = C or G) were used to amplify a segment (35) from the BamHI-W fragment of the EBV genome. Fluorescent energy transfer (FRET) detection probes were commercially synthesized (IT BioChem, Salt Lake City, UT) and labeled with Red 640 as the reporter fluorescent dye: EBVFRETUP.2 (5'-GGCCCAAGGGGGTTCG GTTGAGCTGTAGGCATAGGCTTGGTTATGCCGGTACTG CGGGGCTCTTGGGATATCGTCCATTCCGACAGCAT CGCCAGTCTACTGCTGCCAGCTGCTAGCTATATCGCT TGATGCAATTCTATGTCGCCAGACAGCAATTTGGAG CTCTA, was cloned, and the plasmid was propagated and purified.

The EBV primers described above were used to amplify the mimic (3000 copies) in a separate reaction that included 10 μL of extracted DNA from each patient sample. The mimic detection FRET probes were commercially synthesized (IT BioChem) and labeled with Red 705-CCA GTC ACT ATG GCG TGC TGC TAG-Phosphate-3' and MIMICFRET DN (5'-Red 705-CCA GTC ACT ATG GCG TGC TGC TAG-Phosphate-3'). Real-time PCR was carried out with the LightCycler system, as described above.

To determine the number of human cellular equivalents tested for the presence of EBV, another aliquot of patient DNA samples was also subjected to real-time PCR analysis for the human β-globin gene using the LightCycler Control DNA kit (Roche Molecular Biochemicals), according to the manufacturer’s recommendations.

EBV viral load was calculated by dividing the EBV copy number in a given sample by the number of cellular genome equivalents, based on the human β-globin gene, in the sample. The EBV viral load was expressed per 10^6 cellular genome equivalents.

**Statistical Analysis**

For analysis of the EBV viral load, we used geometric rather than arithmetic means. Therefore, comparisons between groups are in terms of fold changes, not differences. The EBV viral loads per 10^6 genomes were converted to log values (to avoid taking the log of 0, 1 was added to each number before conversion to log values and was subtracted later). The log values were analyzed by analysis of variance. All results were confirmed by analogous nonparametric procedures (Wilcoxon and Kruskal–Wallis tests). All P values are two-sided.
RESULTS

MTX Activation of Lytic EBV Gene Expression in EBV-Transformed Lymphoblastoid Cells and Gastric Carcinoma Cells

We recently found (25,26) that certain chemotherapeutic drugs, including gemcitabine, doxorubicin, cis-platinum, and 5-fluorouracil, can induce the lytic form of EBV infection in latently infected host cells. Therefore, we determined whether MTX can likewise induce the lytic form of EBV infection in an EBV-positive, latently infected gastric carcinoma cell line (AGS-EBV-GFP) or three different EBV-positive LCLs. MTX treatment enhanced the expression of the early viral gene BMRF1 in both cell types using the doses expected in the serum of rheumatoid arthritis patients (Fig. 1, A). In contrast, other drugs commonly used to treat rheumatoid arthritis, including azathioprine, cyclosporine, cyclophosphamide, mycophenolic acid, and prednisone, did not markedly activate expression of BMRF1 in either AGS-EBV-GFP cells (Fig. 1, B) or lymphoblastoid cells (Fig. 1, C), even when used at doses higher than the peak dose expected in patients. Cyclophosphamide may not be metabolized to its active forms in vitro. However, treatment of lymphoblastoid cell lines with 5 μM 4-hydroxycyclophosphamide (a metabolite of cyclophosphamide that is active in vitro) also did not induce lytic EBV gene expression (data not shown). These results indicate that, of the various drugs used to treat rheumatoid arthritis, only MTX induces a substantial increase in lytic EBV gene expression.

Effect of Signal Transduction Pathway Inhibitors on Induction of Lytic EBV Infection by MTX

Previous reports suggested that the PI3 kinase, p38 MAP kinase, and MEK pathways are often required for activation of lytic EBV infection in EBV-positive cell lines treated with various agents (25,36–39). Therefore, we treated three different LCLs with MTX in the absence or presence of inhibitors of PI3 kinase (LY294002), p38 MAP kinase (SB202190), or MEK (PD98059) and analyzed the expression of various EBV lytic proteins in treated cells (Fig. 2). MTX was unable to induce lytic EBV gene transcription in the presence of any of the inhibitors. By contrast, the pan-caspase inhibitor Z-VAD-fmk increased the level of MTX-associated lytic gene induction (Fig. 2), possibly because lytic viral proteins were produced more efficiently when apoptosis was inhibited. The kinase inhibitors showed no cytotoxicity at the
concentrations used (data not shown). These experiments also showed that MTX treatment induced the expression of both EBV IE proteins, BZLF1 and BRLF1, and that this induction was prevented by the three kinase inhibitors. These findings suggest that MTX activates lytic EBV gene expression through its effects on one or both of the EBV IE gene promoters and that the PI3 kinase, p38 MAP kinase, and MEK pathways are required for MTX induction of lytic EBV genes.

Activation of the Two EBV IE Promoters (Rp and Zp) by MTX Treatment

To determine whether MTX treatment results in increased transcription of the BZLF1 (Zp) and BRLF1 (Rp) promoters of EBV, EBV-negative B cells (DG75) were transfected with plasmids containing the wild-type Rp (RpCAT) or Zp (ZpCAT) promoters linked to the CAT gene or with constructs containing site-directed mutations of transcription factor binding sites in each promoter known to be important for the induction of Zp and Rp by other stimuli. Treatment of transfected DG75 cells with MTX more than tripled the CAT activity derived from the ZpCAT construct and more than doubled the CAT activity from the RpCAT construct (Fig. 3) but had no effect on the promoterless control construct (data not shown).

To further define the promoter elements required for the MTX effect on the BRLF1 promoter, we examined CAT expression from an RpCAT construct missing the two Zif268 (EGR-1) binding sites, which have previously been shown to be required for phorbol ester activation of the promoter (30). MTX did not activate the BRLF1 promoter in the absence of the Zif268 binding sites (RpCAT Δzif) (Fig. 3). Similarly, the ZIA and ZIB sites (to which MEF2D binds) (32, 40, 41) and the ZII (CRE) site (to which CREB, ATF-1, ATF-2, and c-jun bind) (36, 42) in the BZLF1 promoter, which have previously been shown to be required for induction of BZLF1 transcription by phorbol esters as well as calcium ionophores (32), were required for MTX induction (Fig. 3). We recently showed that the chemotherapy agents gemcitabine and doxorubicin likewise activate the BRLF1 and BZLF1 promoters through the EGR-1 site in the BRLF1 promoter and the ZI and ZII sites in the BZLF1 promoter (26). Thus, gemcitabine, doxorubicin, and MTX may induce lytic EBV gene transcription by similar mechanisms.

MTX Induction of Lytic EBV DNA Replication in AGS-EBV-GFP Cells

The ability of MTX (and other chemotherapeutic agents) to activate expression of the early lytic viral proteins, which replicate the viral genome during the lytic form of infection, would not necessarily result in replication of the EBV genome, because these drugs might inhibit replication of the EBV genome by the same mechanisms that prevent cellular DNA replication. To determine whether early lytic viral protein expression induced by either gemcitabine or MTX treatment of EBV-infected cells is accompanied by replication of the lytic form of EBV DNA, AGS-EBV-GFP cells were treated with MTX, gemcitabine, or no drug for 72 hours in the presence or absence of the antiviral drug acyclovir. The amount of lytic EBV replication was then analyzed by DNA gel blot analysis using the EBV terminus assay (34), which can distinguish the latent and lytic forms of EBV DNA. The EBV genome persists as an episome (which contains only fused viral termini) during the latent form of infection, whereas lytic viral replication results in an increase in both the fused termini form and a linear form of the viral genome. MTX treatment of AGS-EBV-GFP cells increased the level of both the fused and linear forms of the EBV genome, and this effect was prevented by acyclovir (Fig. 4). In contrast, cells treated with gemcitabine showed no replication of the EBV genome, even though gemcitabine efficiently induced early lytic viral protein expression in the same experiment (data not shown). Thus, although a number of different chemotherapeutic agents activate lytic EBV gene expression, MTX may be unique in its ability not only to activate lytic EBV gene expression but also to activate viral replication.

MTX Induction of the Release of Infectious Progeny Virus From AGS-EBV-GFP Cells

To determine whether MTX treatment induces the complete EBV replicative cycle in host cells, we treated AGS-EBV-GFP cells with MTX, gemcitabine, or no drug in the presence or absence of acyclovir. Immunoblot analysis of the AGS-EBV-GFP extracts showed that MTX and gemcitabine induced similar levels of BMRF1 expression in AGS-EBV-GFP cells in the presence or absence of acyclovir (Fig. 5, A). To determine whether infectious progeny virus was released from AGS-EBV-GFP cells treated with the various drugs, the drug-containing medium was removed and replaced with drug-free medium after 48 hours. The medium from each condition was collected 72 hours after the addition of drug-free medium, passed through a 0.45-μm filter, and used to infect Raji cells. Three days later, Raji cells were harvested to analyze GFP levels by immunoblot-
EBV Loads in Patients With Rheumatoid Arthritis and Association of Immunosuppressive Drug Therapy With release of infectious EBV. Lytic viral gene expression but also markedly enhances the release of latently infected host cells not only results in the induction of EBV-GFP cells resulted in the release of infectious virions. As expected, acyclovir prevented the release of infectious virus from MTX-treated cells. These data suggest that MTX treatment (Fig. 5, B) nor doxorubicin treatment (data not shown) of AGS-EBV-GFP cells (Fig. 5, B). In contrast, neither gemcitabine treatment (Fig. 5, B) nor doxorubicin treatment (data not shown) of AGS-EBV-GFP cells resulted in the release of infectious virions. Ethidium bromide staining of total DNA indicated equal transfer in all conditions (data not shown).

Association of Immunosuppressive Drug Therapy With EBV Loads in Patients With Rheumatoid Arthritis and Polymyositis

Because MTX induced the release of infectious EBV from latently infected cells in vitro, and because patients with rheumatoid arthritis and polymyositis who are treated with MTX have an increased propensity to develop EBV-associated lymphomas, we determined whether rheumatoid arthritis and polymyositis patients treated with MTX have higher EBV loads in their blood than patients on other immunosuppressive regimens. We measured EBV viral loads in the blood of 29 consecutive patients with rheumatoid arthritis and 12 with polymyositis who were receiving immunosuppressive medication. Of the 41 total patients, 29 (23 with rheumatoid arthritis and six with polymyositis) were receiving immunosuppressive medication regimens that included MTX, and 12 (six with rheumatoid arthritis and six with polymyositis) were on immunosuppressive regimens that did not include MTX. Seventy-five percent of patients not receiving MTX received leflunomide, etanercept, mycophenylate mofetil, or cyclosporine, whereas only 21% of patients on MTX received these drugs (Table 1).

EBV-associated lymphomas have not been reported in patients with Wegener granulomatosis; therefore, we measured EBV viral loads in 47 consecutive patients with Wegener granulomatosis as a control. Of these, 30 patients were on regimens that included MTX and 17 were on regimens that did not include MTX. All patients with Wegener granulomatosis not receiving MTX were on mycophenylate mofetil, cyclophosphamide, or azathioprine, whereas no patients receiving MTX were on these medications (Table 1). Use of corticosteroids was higher in patients with Wegener granulomatosis who were not receiving MTX (65%) than in patients who were receiving MTX (13%).

The geometric mean of the EBV viral load in patients with rheumatoid arthritis and polymyositis who were receiving regimens that included MTX was 40 EBV copies per 10⁶ cellular genomes, whereas the EBV viral load in patients on regimens that did not include MTX was 5.1 EBV copies per 10⁶ cellular genomes (geometric mean fold difference in copies, 10.8, 95% confidence interval [CI] = 3.0 to 38; \( P = .011 \)) (Fig. 6). When patients with the two diseases were considered separately, patients with rheumatoid arthritis who received MTX had statistically significantly higher geometric mean EBV copy numbers than those not receiving MTX (mean = 27 EBV copies and 1.8 EBV copies per 10⁶ cellular genomes, respectively; geometric mean fold difference = 9.8, 95% CI = 2.4 to 40; \( P = .011 \)).
however, the difference was not statistically significant in patients with polymyositis, probably due to the small number of patients (n = 12) studied.

In patients with Wegener granulomatosis, among those receiving MTX, the geometric mean EBV copy number was 3.6 EBV copies per 10^6 cellular genomes, whereas among those receiving other immunosuppressive regimens, it was 11 copies per 10^6 cellular genomes; this difference was not statistically significant (geometric mean fold difference = 2.5, 95% CI = 0.7 to 9; P = .12). Although patients with rheumatoid arthritis and polymyositis treated with methotrexate (MTX) had statistically significantly higher geometric mean EBV copy numbers than patients with Wegener granulomatosis who were receiving MTX (mean = 40 EBV copies and 3.6 EBV copies per 10^6 cellular genomes, respectively; geometric mean fold difference = 8.9, 95% CI = 3.4 to 23; P < .001; Fig. 6), the mean weekly dose of MTX in patients with Wegener granulomatosis (18.8 mg) was actually higher than that in patients with rheumatoid arthritis and polymyositis (13.8 mg) (data not shown). The mean length of treatment of patients with Wegener granulomatosis on MTX was 24 months, whereas the mean length of treatment for rheumatoid arthritis and polymyositis patients on MTX was 65 months.

Because corticosteroids were the most common immunosuppressant used in patients with rheumatoid arthritis or polymyositis who were receiving MTX, we compared the geometric mean EBV viral load in patients receiving MTX alone with that in patients receiving MTX and corticosteroids (but no other immunosuppressants). The nine patients with rheumatoid arthritis or polymyositis who received MTX alone had a geometric mean of 29 EBV copies per 10^6 cellular genomes, whereas five patients with these diseases who were receiving MTX and corticosteroids had a geometric mean of 46 EBV copies per 10^6 cellular genomes. Although these numbers are small, the data suggest that corticosteroids had a modest effect on EBV viral load in these patients. The 16 patients with Wegener granulomatosis who were receiving MTX alone had a geometric mean of 2.9 EBV copies per 10^6 cellular genomes, and the three patients with this disease who were receiving MTX and corticosteroids (but no other immunosuppressants) also had a geometric mean of 2.9 EBV copies per 10^6 cellular genomes.

To verify that the differences in EBV viral load were independent of the number of cellular genomes present in a given sample, we compared the number of cellular genomes in patients receiving MTX with those in patients not receiving MTX, as determined by a regression analysis of log viral load on log number of cellular genomes, adjusting for the differences in EBV viral load among the patient groups. The difference in the number of cellular genomes present was not statistically significant in any of the patient groups (data not shown).

**DISCUSSION**

MTX is an effective drug for the treatment of rheumatoid arthritis and polymyositis. Nevertheless, a number of reports have linked the development of EBV-positive lymphomas in patients with rheumatoid arthritis and polymyositis with MTX.

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### Table 1. Immunosuppressive regimens in patients with rheumatoid arthritis (RA), polymyositis (PM), or Wegener granulomatosis

<table>
<thead>
<tr>
<th>Medication</th>
<th>No. of patients receiving immunosuppressive regimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA or PM</td>
<td>Wegener granulomatosis</td>
</tr>
<tr>
<td>Regimens including MTX</td>
<td></td>
</tr>
<tr>
<td>MTX alone</td>
<td>29</td>
</tr>
<tr>
<td>MTX + corticosteroid</td>
<td>9</td>
</tr>
<tr>
<td>MTX + hydroxychloroquine</td>
<td>5</td>
</tr>
<tr>
<td>MTX + corticosteroid + hydroxychloroquine</td>
<td>4</td>
</tr>
<tr>
<td>MTX + corticosteroid + leflunomide</td>
<td>4</td>
</tr>
<tr>
<td>MTX + etanercept</td>
<td>3*</td>
</tr>
<tr>
<td>Others</td>
<td>12</td>
</tr>
<tr>
<td>Regimens not including MTX</td>
<td></td>
</tr>
<tr>
<td>Corticosteroid</td>
<td>2</td>
</tr>
<tr>
<td>Corticosteroid + mycophenylate mofetil</td>
<td>2</td>
</tr>
<tr>
<td>Corticosteroid + cyclophosphamide</td>
<td>0</td>
</tr>
<tr>
<td>Mycophenolate mofetil</td>
<td>0</td>
</tr>
<tr>
<td>Others</td>
<td>8‡</td>
</tr>
</tbody>
</table>

*One patient each: MTX + corticosteroid + mycophenylate mofetil; MTX + corticosteroid + azathioprine; MTX + corticosteroid + etanercept.
†One patient each: MTX + cyclophosphamide; MTX + leflunomide; MTX + azathioprine; MTX + etanercept + hydroxychloroquine; MTX + cyclosporine; MTX + prednisone; MTX + daclizumab.
‡One patient each: hydroxychloroquine; etanercept; corticosteroid.
§One patient each: corticosteroid + azathioprine; azathioprine.

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**Fig. 6.** Epstein–Barr virus (EBV) DNA load in patients with rheumatoid arthritis and polymyositis treated with methotrexate (MTX). EBV DNA levels in peripheral blood mononuclear cells from patients with rheumatoid arthritis (RA) and polymyositis (PM) (left panel) and patients with Wegener granulomatosis (right panel) were compared in patients who had MTX-containing and non–MTX-containing regimens. The horizontal lines indicate the geometric mean EBV copy number for each group. The number of EBV copies in a patient sample was determined by quantitative polymerase chain reaction, and the number of cellular genomes was calculated by polymerase chain reaction for the human β-globin gene in the sample. The EBV viral load was expressed as the number of EBV copies per 10^6 cellular genomes.
treatment (4–6,9,21,22). Moreover, MTX withdrawal can result in regression of some EBV-positive lymphomas in patients with rheumatoid arthritis and polymyositis, further suggesting that the drug contributes directly to the development of these lymphomas. In this article, we demonstrated that MTX induces reactivation of EBV from latent infection in two types of host cells and leads to the release of infectious virions. Furthermore, we showed that patients with rheumatoid arthritis or polymyositis who were treated with an MTX-containing regimen had statistically significantly higher EBV loads than patients on immunosuppressive regimens that did not include MTX. Because the EBV loads measured in this study were derived from intracellular (rather than extracellular) virus, we cannot exclude the possibility that the MTX-associated increase in EBV load was due primarily to the immunosuppressive effect of the drug allowing proliferation of latently infected cells rather than to any effect of the drug on viral replication. However, the former seems unlikely, given that treatment of patients with other immunosuppressive drugs was not associated with an increased intracellular EBV load. Our data suggest that the unique ability of MTX to induce EBV replication while simultaneously promoting immunosuppression may explain the association of this drug with the development of EBV-positive lymphomas in patients with rheumatoid arthritis or polymyositis.

EBV transforms primary B cells in vitro and plays an important role in the pathogenesis of most B-cell lymphomas arising in immunocompromised patients. In healthy individuals, EBV persists in a latent form in a very small number of B cells (approximately one in 10^6 cells) for the life of the host, and it is difficult to detect EBV in the plasma of healthy individuals, even using extremely sensitive methods such as PCR (43). In immunocompetent hosts, EBV does not normally cause B-cell lymphomas, presumably because circulating EBV-specific cytotoxic T cells control the number of latently infected B cells. However, T cells from patients with rheumatoid arthritis have defects in their response to EBV-infected cells in vitro (44,45) and thus such patients may be more susceptible than healthy individuals to EBV-induced lymphomas, even in the absence of immunosuppressive agents. Interestingly, rheumatoid arthritis patients have an abnormally high number of circulating EBV-positive B cells (46). Furthermore, the constant stimulation of B cells in this disease may also increase the propensity of rheumatoid arthritis patients to develop B-cell lymphomas (47,48). Given the transforming capacity of EBV, the known defect in the immune response of rheumatoid arthritis patients to EBV, and the additional immunosuppressive effect of MTX, it has been generally assumed that these factors together are sufficient to explain why a small number of rheumatoid arthritis patients treated with MTX develop EBV-positive lymphomas.

However, many drugs other than MTX that are equally immunosuppressive have not been associated with an increased number of EBV-positive lymphomas in rheumatoid arthritis patients. Therefore, we explored the hypothesis that MTX specifically activates the lytic phase of EBV replication in latently infected host cells leading to release of infectious virus, because an increased level of infectious EBV in immunosuppressed rheumatoid arthritis patients might overwhelm the capacity of the cytotoxic T cells in these patients to eliminate early EBV-positive tumor cells. EBV-positive lymphomas contain the latent, rather than lytic, form of EBV infection. Nevertheless, a high level of circulating infectious EBV in immunosuppressed patients would be expected to increase the number of latently infected B cells as well as the likelihood that an EBV-infected B cell would progress to a lymphoma.

In this study, patients with rheumatoid arthritis and polymyositis whose immunosuppressive regimens included MTX had statistically significantly higher EBV DNA loads in the blood than patients on other immunosuppressive regimens. Previous reports indicating that EBV-associated lymphoproliferative disease in patients with rheumatoid arthritis and polymyositis occurs predominantly in those who receive MTX (4–6), suggested that MTX may result in a dysregulation in the control of EBV. Our results here suggest that MTX may allow increased reactivation of virus in addition to impairing the immune response to EBV. The observation that MTX treatment resulted in higher EBV loads than other immunosuppressants, combined with the finding that MTX induced production of EBV from latently infected cells, suggests that increased reactivation of EBV is involved in the development of MTX-associated lymphomas.

Our results differ from those of another recent study in which the EBV load of 33 rheumatoid arthritis patients treated with MTX was not statistically significantly higher than that in patients treated with other regimens (49). However, in that study, the majority of patients received only one immunosuppressant medication, whereas 65% of the patients in our study who were receiving MTX for rheumatoid arthritis were also on other immunosuppressants (most frequently corticosteroids). Thus, the patients in our study may have had more severe disease and/or been more immunosuppressed than those in the previous study, resulting in higher MTX-associated EBV viral loads in this study.

In contrast to the effect of MTX treatment in patients with rheumatoid arthritis and polymyositis, patients with Wegener granulomatosis whose immunosuppressive regimens included MTX did not have a higher EBV DNA load in their blood than patients on regimens that did not include MTX. This result is consistent with the observation that EBV lymphoproliferative disease has not been reported in patients with Wegener granulomatosis. Thus, factors in addition to MTX are likely to be important for elevation of EBV DNA load and development of lymphoproliferative disease in patients with rheumatoid arthritis and polymyositis. One possibility is that the host immune response directed against lytically infected EBV-positive cells results in rapid elimination of these cells in normal individuals and in patients with Wegener granulomatosis but not in patients with rheumatoid arthritis and polymyositis. In addition, the chronic immune stimulation present in patients with autoimmune diseases (e.g., rheumatoid arthritis and polymyositis) may increase the susceptibility to malignancy or result in proliferation of an abnormal B-cell clone that ultimately becomes malignant. The elevated EBV DNA loads and risk of EBV-associated lymphoproliferative disease observed in patients with rheumatoid arthritis and polymyositis are likely due to both the immunosuppressive regimen and the underlying immunologic abnormalities specific to these diseases.

EBV infection is usually latent in B cells but can be switched to the lytic form of infection in vitro using a variety of agents, including phorbol ester (50), TGF-beta 1 (37), calcium ionophores (51), n-butyrate (52,53), cross-linking of surface immunoglobulin (54), and 5-azacytidine (55). All of these stimuli share the ability to activate transcription of the two EBV IE genes, BZLF1 and BRLF1. BZLF1 and BRLF1 encode tran-
scriptional activators, and overexpression of either protein under the control of a strong heterologous promoter is sufficient to activate the lytic form of infection (1, 2, 54, 56, 57). As with those other stimuli, MTX activates the lytic form of EBV infection through its effects on the BZLF1 and BRLF1 promoters. Moreover, the same transcription factor binding motifs (EGR-1, MEF2, and CRE) that have been shown to be essential for the activating effects of other viral inducing agents (30–32) were also required for the MTX effect on the BZLF1 and BRLF1 promoters. Our data indicate that the ability of MTX to induce lytic EBV gene expression requires several signal transduction pathways (PI3 kinase, p38 MAP kinase, and MEK) that are required for lytic viral infection following ligation of the B-cell receptor (36, 38, 39) or treatment with TGF-beta 1 (37). The requirement for the p38 MAP kinase pathway may reflect the ability of this kinase to activate both the c-jun and ATF-2 transcription factors (60). Furthermore, several chemotherapeutic agents have been shown to activate EGR-1 through both p38-dependent and MEK-dependent mechanisms (61, 62).

We recently reported (25, 26) that several different chemotherapeutic agents can activate lytic EBV gene transcription in host cells via mechanisms similar to those we found in this study to be used by MTX. The ability of EBV to use signal transduction pathways that are routinely activated during host cell stress as a mechanism to convert from the latent to the lytic form of viral infection may allow the virus to escape from a dying host cell and subsequently reinfect a healthy cell. At present, MTX is unique among the other agents tested in its ability to also induce the release of infectious EBV. It is not surprising that chemotherapeutic agents that inhibit cellular DNA replication likewise inhibit EBV DNA replication. Indeed, gemcitabine may be effective as an anti-EBV agent (Feng WH, Kenney SC; unpublished data). It is perhaps surprising that lytic EBV replication is not inhibited by MTX, a dihydrofolate reductase inhibitor that inhibits cellular DNA synthesis by interfering with the de novo thymidine synthesis pathway. One possible explanation is that the EBV-encoded thymidine kinase expressed during lytic infection may allow the virus to bypass the inhibitory effect of MTX by increasing the synthesis of dTMP via the salvage pathway.

In summary, we have demonstrated that MTX activates lytic EBV infection, resulting in the release of infectious virus from host cells. The ability of MTX to increase the level of infectious EBV in patients, combined with its potent immunosuppressive effects, may collaborate to induce EBV-positive lymphomas in patients with rheumatoid arthritis and polymyositis, who already have a high risk of developing B-cell lymphomas than healthy individuals. Our data raise the possibility that the antiviral drug acyclovir, if given simultaneously with MTX, might reduce the number of MTX-associated lymphomas in patients with rheumatoid arthritis and polymyositis. Nevertheless, it is important to note that the number of MTX-associated lymphomas in rheumatoid arthritis and polymyositis patients is extremely low, with a yearly incidence of less than one in 1000 rheumatoid arthritis patients treated with MTX (63); thus, antiviral treatment to reduce the number of MTX-associated lymphomas, even if effective, might not be justified. Whether MTX treatment of other groups of patients, such as cancer patients, has any unintended consequences due to EBV reactivation remains an unexplored issue. Future studies should explore whether MTX treatment of other patients, such as those with malignancies, may increase the risk of EBV reactivation and other EBV-associated tumors.

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NOTES

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