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EBV Suppresses Prostaglandin E₂ Biosynthesis in Human Monocytes

Martin Savard,* Carole Bélanger,* Michel J. Tremblay,† Nancy Dumais,† Louis Flamand,‡ Pierre Borgeat,* and Jean Gosselin*‡

It is well known that EBV has developed strategies to evade immune surveillance. Previously, EBV was shown to bind specifically to monocytes and regulate expression of proinflammatory mediators such as IL-1, IL-6, TNF-α, and leukotrienes. EBV was also found to affect phagocytosis of monocytes. In this study, we show that in addition to these effects, EBV suppresses the biosynthesis of PGE₂, a pleiotropic immunomodulatory molecule that is synthesized by the dioxygenation of arachidonic acid via the cyclooxygenase (COX) pathway. This down-regulation of PGE₂ formation involved the inhibition of the inducible COX-2 isoform expression both at the transcriptional and translational levels, whereas expression of the constitutive COX-1 isoform was unaltered. Furthermore, exposure of monocytes to EBV was found to impact on the NF-κB activation pathway, which plays an essential role in the induction of COX-2 in monocytes. The inhibition of PGE₂ biosynthesis was relieved when the experiments were conducted in presence of phosphonoacetic acid, an inhibitor of herpesviruses DNA polymerase, indicating that viral replication and/or neosynthesized viral proteins were involved in this process. Thus, inhibition of PGE₂ biosynthesis in monocytes may represent an additional mechanism underlying EBV pathogenicity.

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Prostaglandins constitute a family of potent proinflammatory lipidic compounds derived from arachidonic acid metabolism. Together with leukotrienes, which form another group of arachidonate derivatives, they are involved in the control of inflammatory and immune functions. PGs are generated by the dioxygenation of arachidonic acid via the cyclooxygenase (COX)³ pathway (1). Two distinct isoforms of COX have been described: COX-1, which is constitutively expressed in most tissues, and COX-2, whose expression is transiently induced by a variety of stimuli, including cytokines, growth factors, and phorbol ester. It is well established that the inducible isoform COX-2 is responsible for the biosynthesis of PGE₂ by activated monocytes/macrophages (2, 3).

PGE₂ is a potent vasodilator (4, 5) that also prevents overactivation of cellular immunity (6–8). It was also reported that PGE₂ exerts antiviral activities. For example, in vitro treatment of monocyte-derived macrophages with PGE₂ decreased HIV-1 virions penetration by suppressing expression of the HIV-1 coreceptor CCR5 (9). As well, addition of exogenous PGE₂ was shown to reduce replication of adenoviruses types 1, 5, and 12, and parainfluenza virus presumably by increasing intracellular levels of cAMP (10, 11). PGE₂ antiviral action has also been reported in vivo. Successive treatments of liver-transplanted patients suffering from recurrent hepatitis B virus infection, with i.v. PGE₁ and oral PGE₂, led to a significant decrease of viral replication (12).

The EBV, which belongs to the Herpesvirus family, has demonstrated its capabilities to adapt and evade host defense mechanisms. For instance, latent infection of B lymphocytes in vivo is one effective mean used by EBV to evade immune surveillance (13). The EBV BCRF-1 gene encodes a homologue of IL-10, which, like its cellular counterpart, possesses immunosuppressive properties (14). EBV is also able to interact with phagocytes such as neutrophils and monocytes and modulate synthesis of proinflammatory mediators (15–17). Moreover, our laboratory has previously shown that neutrophils are permissive to EBV viral entry and that EBV-infected neutrophils undergo premature cell death by apoptosis (18). More recently, we reported that EBV replicates in monocytes and reduces the ability of these cells to phagocytose (19). Impairment of the phagocytosis machinery is likely to be advantageous for the viral outcome, because phagocytosis is involved in elimination of foreign organisms. In the present study, we show that EBV affects another cellular function of monocytes, the biosynthesis of PGE₂. Because PGE₂ is an important immunoregulatory mediator, this suppressive effect of EBV on its formation may represent an important event in the disruption of the immune response during the early stages of infection and may constitute another viral strategy to evade the immune surveillance.

Materials and Methods

Isolation of monocytes

PBMCs obtained from healthy volunteers were isolated by centrifugation of heparinized venous blood over Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden). PBMCs were next submitted to a Percoll density centrifugation to separate the monocytes from the lymphocyte populations, as previously described (20), and were further enriched using a cell-sorting
procedure (EpicS Elite ESP, Coulter Electronics Canada, Burlington, Ontario, Canada). This resulted in 99% pure monocyte suspensions, as assessed by flow cytometry using anti-CD14 mAb (Becton Dickinson, Mississauga, Ontario, Canada). Cell viability was tested by the trypan blue dye exclusion procedure and was superior to 99%.

Viral preparations

Preparations of EBV (strain B95-8) were produced as described previously (17). Briefly, B95-8 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, at 37°C in a humidified atmosphere with 5% CO2. Culture medium was harvested at the indicated times and stored at −80°C until use. Cell tiers were evaluated and adjusted to 10^6 transforming units (TFU/ml), as previously described (21). For every experiment, cell-free supernatants collected from B95-8 cells that have not been exposed to EBV were processed as described above and served for mock-infected controls. When mentioned, EBV virions were inactivated by heating (1 h, 56°C), or by exposure to UV radiation (265 nm, 60 min), or by neutralization with the mAb 72A1 against the viral envelope gp350/220, as described elsewhere (16).

Culture conditions

Enriched monocytes (1 × 10^6 cells/ml) were preincubated for 1 h at 37°C with either infectious (10^6 TFU/ml) or inactivated EBV and then stimulated with 1 µg/ml of LPS (Sigma, St. Louis, MO) for time periods ranging from 1 to 24 h. In some cases, enriched monocytes were preincubated for 1 h with 200 µg/ml of phosphonoacetic acid (PAA), an inhibitor of viral DNA polymerase, before treatment with infectious EBV. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, at 37°C in a humidified atmosphere with 5% CO2. Culture medium was found to contain less than 10 pg/ml of endotoxins, as evaluated by the Limulus amebocyte lysates. Cell-free supernatants from each culture were harvested at the indicated times and stored at −80°C until used for PGE2 assays.

Enzyme immunoassays for PGE2, IL-1β, and IL-6

Supernatants from mock-treated and EBV-treated monocyte cultures were harvested at the indicated times and tested for the presence of PGE2, IL-1β, and IL-6, using commercially available enzyme immunoassays with acetylcholine esterase as label (Cayman Chemical, Ann Arbor, MI). The detection limit for PGE2 was 29 pg/ml, with less than 0.01% cross-reactivity for other PGs. The detection limit for both IL-1β and IL-6 was 1.5 pg/ml without significant cross-reactivity with other known cytokines.

Analysis of CD14 expression by flow cytometry

Monocytes (1 × 10^6 cells) were infected with EBV particles (Fig. 2) and, at the indicated times, cells were washed twice with cold PBS (pH 7.4), followed by a preincubation for 15 min at 4°C with 15 µg of human IgG (Miles Canada, Ontario, Canada) in 100 µl of PBS to block Fc receptors. Cells were then washed with PBS and incubated for an additional 30 min at 4°C with either a fluoresceinated FITC anti-human CD14 mAb, or an IgG1 negative control FITC conjugate (BioSource International, Camarillo, CA). After washing and fixation in 0.5% paraformaldehyde, cells were analyzed with an EPICS-XL flow cytometer (Coulter Electronics) at an excitation setting of 488 nm and emission setting of 540 nm. Fluorescence was gated from monocytes, as judged by their forward and side scatter.

Western blot analysis

At the indicated times, untreated and LPS- and EBV-treated monocytes were washed twice with PBS (pH 7.4) and lysed in 100 µl of ice-cold HBSS containing 0.5% Triton X-100 and a cocktail of antiproteases consisting of 100 µM PMSE, 10 µg/ml leupeptin, and 10 µg/ml aprotinin. Samples were diluted in 2× Laemmli buffer, boiled, electrophoresed on a 10% SDS-polyacrylamide gel, and immunoblotted, as described previously (16). Briefly, proteins were transferred for 3 h at 500 mA current setting onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA) and then incubated for 1 h at room temperature in blocking solution containing anti-COX-1 (1/5000 diluted) or anti-COX-2 (1/2500 diluted) antisera (kindly provided by Dr. Jacques Maclouf, Institut National de la Sante et de la Recherche Medicale, Hôpital Lariboisiere, Paris, France). Membranes were washed twice with TBS-Tween (0.15% v/v) and treated with a HRP-linked donkey anti-rabbit Ab. Bound Abs were visualized using the enhanced chemiluminescence (ECL) reagent according to the manufacturer’s protocol (Life Science, Boston, MA).

Northern blot analysis

Total RNA from unstimulated, EBV-treated, and LPS-stimulated monocytes (1 × 10^6 cells) was isolated using TRIzol reagent according to the manufacturer’s instructions (Life Technologies, Burlington, Ontario, Canada). Ten micrograms of RNA were loaded on a 1% agarose/formaldehyde gel and size fractionated by electrophoresis. RNA was then transferred overnight by capillary diffusion onto a Hybond-N nylon membrane (Amersham Canada Limited, Oakville, Ontario, Canada). The membranes were first preincubated for 3 h at 42°C, in a buffer containing 5% standard saline citrate and 200 µg/ml polyethyleneimine (100 µg/ml), 5% dextran sulfate, and 100 µg/ml salmon sperm DNA. The hybridization was performed overnight in the same buffer containing 1 × 10^6 cpm/ml of the 32P-labeled cDNA probes (Prime-a-Gene labeling system) specific for COX-2 (1.3 kb), IL-1β (1.3 kb), and IL-6 (1.2 kb) (the specific cDNA fragments were kindly provided by Dr. Patrice Poubelle, Centre de Recherche en Rhumatologie et Immunologie, Centre Hospitalier de l’Université Laval, Québec, Canada). Membranes were subsequently washed twice in 0.5 × SSC, 0.1% SDS at 42°C for 30 min, followed by a more stringent wash at 65°C for 30 min in the same solution. Membranes were exposed to Kodak X-OMAT films at −80°C with intensifying screens. The GAPDH cDNA probe was used as internal control to demonstrate equal loading of RNA in each lane. The intensity of each autoradiographic signal was quantified by laser densitometry (FujiFilm, BAS-1800) and was normalized to its respective GAPDH signal.

Nuclear extract preparation and EMSA

Uninfected and infected monocytes were treated with LPS (1 µg/ml) for 30 min, and levels of nuclear NF-κB were measured by EMSA. Nuclear extracts were prepared as described elsewhere (22). For EMSA, 5 µg of nuclear extracts were incubated with 1 ng of 32P-labeled double-stranded oligonucleotide, containing either the consensus binding site for NF-κB/c-Rel homodimeric and heterodimeric complexes (underlined), 5′-AGTT GACCAAGCTTTCGCGGAC-3′, or as controls, the mutated NF-κB site 5′-AGTTGACGCTTTCGCGGAC-3′, or a scrambled sequence that contains in this case the CREB DNA binding motif 5′-AGAGTGTCCGGACGTCAGAGGACTTAG-3′ (Santa Cruz Biotechnology, Santa Cruz, CA). The oligonucleotides were 5′ end labeled with [γ-32P]ATP using T4 polynucleotide kinase (Promega, Madison, WI), and unincorporated 32P labels were removed on a G-50 Sephadex column. The incubation was performed at room temperature for 30 min in 1× binding buffer (20 mM HEPES, pH 7.9, 250 mM KCl, 5 mM EDTA, 250 mM NaCl, 10% Ficoll, 40% glycerol, 10 mM DTT) containing 2 µg of poly(dI-dC) and 10 µg of nuclease-free BSA. As an additional control, nuclear extracts from LPS-stimulated monocytes were preincubated for 30 min with 100-fold excess of unlabeled NF-κB oligonucleotide, which completely abolished binding of the radiolabeled oligonucleotide to the nuclear proteins. Samples were subjected to electrophoresis on a 4% nondenaturing polyacrylamide gel for 1.5 h at 150 V in Tris-borate-EDTA (TBE) 0.5× buffer. Gels were subsequently dried and exposed to Kodak X-OMAT films at −80°C with intensifying screens.

Results

Inhibition of PGE2 biosynthesis by EBV

We have previously reported that EBV affects arachidonate metabolism in monocytes through priming for 5-lipoxygenase product generation in response to stimulation with a second agonist (16). To establish whether EBV can also interfere with the COX metabolism in monocytes through priming for 5-lipoxygenase products, we performed EMSA (Fig. 1). However, when monocytes were preincubated for 1 h with EBV, and subsequently stimulated with LPS, a reduction in PGE2 biosynthesis was observed. Such a suppression of PGE2 biosynthesis was significant from 12 h poststimulation with a 60–65% reduction in PGE2 expression (Fig. 1). In contrast, the basal levels of PGE2, which are mainly produced by the constitutive COX-1,
remained low throughout the time course and were not significantly affected by exposure to the virus. Taken together, these results suggest that EBV may selectively suppress PGE2 generation via the inducible COX-2 pathway.

To ensure that EBV does not affect LPS responsiveness simply by interfering with CD14, which serves as a receptor for LPS (23), we compared the levels of membrane-bound CD14 molecules in uninfected vs EBV-infected monocytes. As seen in Fig. 2A, control monocytes or monocytes that were incubated on ice with EBV to allow virus-cell interactions showed similar levels of CD14 receptors, suggesting that EBV does not bind to CD14, nor interferes with the availability of CD14 receptors. Moreover, levels of CD14 in infected cells remained comparable with those in control monocytes even after 24- and 48-h exposure to EBV at 37°C (Fig. 2, B and C), which allows expression of EBV viral genes in monocytes (19).

Effect of EBV on the expression of COX-2 in human monocytes
As a first step toward understanding the nature of the EBV-mediated suppressive action, we evaluated its effect on COX-2 expression, an enzyme involved in PGE2 biosynthesis. This isoform of COX, normally absent in resting monocytes, can be induced by a variety of stimuli, including LPS. As depicted in Fig. 3, freshly isolated monocytes do not constitutively express the COX-2 protein. However, COX-2 levels increased rapidly following LPS stimulation, with maximal expression at 12 h poststimulation with LPS. As expected, levels of the noninducible COX-1 isoform were not affected by LPS stimulation. The doublet observed in Western blot analysis is characteristic of the COX-2 isoform and is due to glycosylation of the protein (24). These results are consistent with previous studies that have attributed the enhanced production of PGE2 in stimulated monocytes/macrophages to the inducible and transiently expressed COX-2 isof orm (25). Monocytes pretreated with EBV showed reduced levels of inducible COX-2 protein at 8 h poststimulation with LPS (Fig. 3), which corresponds to the time point at which PGE2 levels began to be suppressed by EBV (Fig. 1). Maximal effects of EBV were observed between 12 and 24 h, at which times expression of COX-2 was barely detectable. This reduction of COX-2 protein levels coincided with reduced levels of COX-2 mRNA transcripts by Northern blot analysis. As shown in Fig. 4, freshly isolated monocytes (0 h) express scarce amount of COX-2 mRNA. Four hours after LPS stimulation, COX-2 mRNA was easily detectable with minimal inhibition caused by EBV. However, by 8 h poststimulation with LPS, COX-2 mRNA levels were reduced by EBV treatment by more than half, as determined by densitometric analysis. Similar reduction in COX-2 mRNA levels was observed at 12 h, and maximal inhibition was observed at 24 h poststimulation. Thus, it appears that reduction in PGE2 biosynthesis by EBV-treated monocytes is directly related to a reduction in COX-2 mRNA levels.
Effects of EBV on COX-2 protein expression in human monocytes. Monocytes were preincubated for 1 h at 37°C in the presence or absence of infectious EBV (10^5 TFU/ml) and stimulated or not with LPS (1 μg/ml) for the indicated times. The expression of both COX-1 and COX-2 proteins was monitored over a 24-h period by immunoblotting, using specific anti-COX-1 and anti-COX-2 antisera, as described in Materials and Methods. The mock-infected control represents monocytes that were exposed to cell-free supernatant obtained from resting B95-8 cells, as described in Materials and Methods. Results shown are representative of three different experiments.

**Effect of inactivated virus on COX-2 expression**

Additional experiments were then performed using inactivated virions and an inhibitor of viral DNA polymerase to define the mechanism through which EBV can diminish PGE_2 formation in activated monocytes. Because maximum inhibitions were observed 24 h after addition of LPS, we examined COX-2 expression at this time point. Infectious EBV significantly reduced LPS-induced COX-2 protein levels (Fig. 5). To prevent specific binding of virions to the cell surface, viral particles were either heat inactivated or neutralized with the mAb 72A1, which recognizes the external viral envelope gp350/220. In both cases, EBV lost its suppressive effect on COX-2 expression (Fig. 5). Since we recently demonstrated EBV replication in monocytes (19), we were also interested in the potential involvement of neosynthesized viral proteins in the mediation of the EBV effects on PGE_2 biosynthesis. To assess this hypothesis, EBV virions were inactivated by UV irradiation, which causes DNA damages and prevents viral gene transcription with minimal effects on viral particle structural integrity and infectivity. UV-treated EBV could not suppress LPS-induced COX-2 expression (Fig. 5), suggesting that binding to the cell surface and entry of virus into the monocytes are not sufficient to cause COX-2 inhibition. These results strongly suggest that viral gene transcription is needed to observe a reduction in COX-2 levels. In an attempt to determine whether early or late EBV proteins are playing a role in the noticed COX-2 inhibition, we performed experiments in the presence of PAA, a viral DNA polymerase inhibitor that allows expression of early EBV proteins while preventing late protein synthesis. As shown in Fig. 5, in the presence of PAA, EBV was no longer able to suppress COX-2 protein expression, suggesting that a late EBV protein is likely to be involved in COX-2 inhibition.

**EBV induces activation of other proinflammatory mediators**

To verify whether the suppressive effect of EBV on PGE_2 synthesis was merely due to a general shutdown of host protein synthesis caused by the virus infection, we examined the impact of EBV on other LPS-inducible proinflammatory mediators such as the cytokines IL-1β and IL-6. As shown in Fig. 6A, incubation of monocytes with EBV stimulated secretion of IL-6, and to a lesser extent IL-1β, at 24 and 48 h postinfection. The potent IL-6 induction by EBV was also observed at the transcriptional level, in which expression of IL-6 mRNA was increased by 2–3-fold in infected cells as compared with untreated cells (Fig. 6B). These effects of EBV on IL-1β and IL-6 are in contrast to the PGE_2 synthesis, in which no induction was observed following the same time course (see Fig. 1). The fact that EBV can specifically induce expression and secretion of IL-1β and IL-6 revealed that there is no overall shut-down of host protein synthesis.

Because EBV repressed the levels of LPS-inducible COX-2, we examined the effects of EBV on LPS responsiveness of IL-1β and IL-6. As shown in Fig. 6B, incubation of monocytes with EBV stimulated secretion of IL-6, and to a lesser extent IL-1β, at 24 and 48 h postinfection. The potent IL-6 induction by EBV was also observed at the transcriptional level, in which expression of IL-6 mRNA was increased by 2–3-fold in infected cells as compared with untreated cells (Fig. 6B). These effects of EBV on IL-1β and IL-6 are in contrast to the PGE_2 synthesis, in which no induction was observed following the same time course (see Fig. 1). The fact that EBV can specifically induce expression and secretion of IL-1β and IL-6 revealed that there is no overall shut-down of host protein synthesis.
IL-6. As described above, EBV does not seem to modulate expression of CD14, a known LPS receptor (see Fig. 2). As expected, LPS stimulated expression of IL-1β and IL-6 both at the mRNA level (Fig. 6B) and protein level (data not shown). However, in contrast to COX-2 expression, the presence of EBV did not affect LPS induction of those cytokines (Fig. 6B), suggesting that LPS responsiveness of monocytes is not overall affected. Therefore, EBV can differentially modulate expression of proinflammatory mediators, presumably by affecting different transductional pathways.

Inhibition of NF-κB translocation in EBV-infected cells
Among the few transductional mechanisms that are currently known to be implicated in COX-2 gene regulation, activation of the NF-κB pathway is of particular importance in monocytes/macrophages (26, 27). In resting cells, NF-κB is normally present in the cytoplasm in its inactivated form, complexed with its inhibitor, IκB. Upon stimulation, NF-κB is dissociated from IκB, and translocates to the nucleus, where it mediates its transcriptional activity through binding to consensus DNA sequences (GGGNNTNCCC) found within the COX-2 promoter. We therefore investigated the possibility that EBV may cause inhibition of COX-2 expression by interfering with the nuclear translocation of NF-κB. Thus, monocytes infected or not with EBV for 1 h were stimulated with LPS, a stimulus known to promote translocation of NF-κB from the cytoplasm to the nucleus. The presence of the transcription factor inside the nuclei was determined by incubation of nuclear extracts with a radiolabeled oligonucleotide probe, containing a NF-κB recognition sequence, followed by nondenaturing gel electrophoresis. The NF-κB-specific complexes are indicated by two arrows. Lane 6. Competition experiment performed in presence of 100-fold molar excess of unlabeled NF-κB oligonucleotide to abolish specific binding. These results are representative of three separate experiments.

FIGURE 6. Effects of EBV on production of secreted cytokines IL-1β and IL-6. Enriched monocytes were preincubated with or without EBV for 24 h and cultured for the indicated times. A, Levels of IL-1β and IL-6 in cell-free supernatants from EBV-infected cultures were evaluated by enzyme immunoassays, as described in Materials and Methods. The mock-infected controls were subtracted from each value shown in the histogram. Results are the means ± SD from two separate experiments. B, Analyses of IL-1β and IL-6 mRNA expression were evaluated by Northern blot analysis at 24 h poststimulation, as described in Materials and Methods. The mock-infected control represents monocytes that were exposed to cell-free supernatant obtained from resting B95-8 cells, as described in Materials and Methods. GAPDH mRNA present in each sample was used as an internal control to assess the quantity of RNA loaded.

FIGURE 7. Effects of EBV on NF-κB translocation in human monocytes. Cells were treated or not with EBV for 1 h and, when indicated, stimulated with LPS (1 μg/ml) for 30 min at 37°C. Cells were washed and nuclear extracts were obtained, as described in Material and Methods. 32P-labeled oligonucleotides containing either a consensus NF-κB sequence (lanes 2–6), a mutated NF-κB site (lane 7), or a scrambled sequence (lane 8) were incubated for 30 min at room temperature with 5 μg of nuclear extracts, followed by nondenaturing gel electrophoresis. The NF-κB-specific complexes are indicated by two arrows. Lane 6. Competition experiment performed in presence of 100-fold molar excess of unlabeled NF-κB oligonucleotide to abolish specific binding. These results are representative of three separate experiments.
competition with unlabeled NF-κB oligonucleotide, or by incubating nuclear extracts with a labeled oligonucleotide containing a mutated NF-κB site, or a CREB site. In all cases, no specific NF-κB complexes could be detected. As expected, addition of LPS induced the levels of nuclear proteins capable of binding to NF-κB consensus sequence. However, LPS stimulation of EBV-infected monocytes did not cause NF-κB translocation to the nucleus, suggesting that the inhibition of COX-2 expression could be attributable by the failure to effectively activate NF-κB in EBV-infected cells.

Discussion

There is growing evidence supporting that infection of monocytes/macrophages by herpesviruses may represent a generalized evolutionary advantage for ensuring propagation and persistence within the host. In the case of EBV, our laboratory first reported that a specific interaction between the virus and monocytes leads to a modulation of proinflammatory mediators such as induction of IL-1, IL-6, and leukotrienes (16, 28), and suppression of TNF-α synthesis (29). More recently, we observed that EBV could replicate and establish a complete lytic cycle in freshly isolated monocytes (19). In the present study, we show that EBV suppresses PGE₂ biosynthesis in LPS-activated monocytes. PGE₂ inhibition by EBV results from repression of COX-2 mRNA synthesis. As anticipated, COX-1 levels were not affected by either LPS or EBV treatment, confirming the role of COX-2 as the major isoform involved in PG synthesis induced by inflammatory stimuli. A specific interaction between EBV and the cell surface of monocytes appears necessary for COX-2 inhibition, because virions preincubated with the neutralizing mAb 72A1 (raised against the viral envelope gp350/220) could not suppress COX-2 expression. Although EBV binds to B lymphocytes via CD21, its receptor on monocytes remains to be identified (15). We have investigated the possibility that EBV might bind to CD14, which serves as a receptor for LPS. However, treatment of monocytes with Abs against CD14 did not prevent viral adsorption to the cell surface, suggesting that EBV binding to monocytes does not involve CD14 (unpublished data). In the present study, we show that EBV binding and infection do not interfere with CD14 molecule. Therefore, the inhibition of COX-2 expression may be caused by occupancy of CD14 by EBV, which would prevent LPS interaction with its receptor, is unlikely. Furthermore, preincubation of monocytes with EBV does not affect LPS responsiveness of IL-6, suggesting that EBV-infected monocytes possess functional LPS receptors. Thus, the suppressive effect of EBV on PGE₂ synthesis is likely to occur at steps downstream of the LPS receptor binding, possibly through interference with signaling proteins such as protein tyrosine kinases and protein kinase C, which are known to be involved in the induction of COX-2, TNF-α, and IL-1β gene expression (30–33). Interestingly, we observed that PMA-induced PGE₂ biosynthesis in monocytes is also inhibited by EBV, indicating that the suppressive effect of the virus on PGE₂ formation is not specific to LPS-activated monocytes.

Interestingly, EBV also lost its suppressive action on COX-2 expression when monocytes were incubated with viral particles inactivated by heat denaturation, UV irradiation, or when monocytes were preincubated with phosphonoacetic acid, an inhibitor of herpesviruses DNA polymerase. These results confirm that both structural integrity and infectivity are required to mediate EBV-induced suppression, and suggest that neosynthesized viral proteins are responsible for this process. That the suppression of both COX-2 expression and PGE₂ release by EBV required a minimum of 8 h of infection might indicate that the suppression involves viral genes expressed late during infection. Which viral protein(s) is (are) responsible for the reduction in COX-2 transcription remains to be determined.

Although COX isozymes are essential components of the cellular enzymatic machinery involved in the biosynthesis of PGs, other enzymes, in particular the cytosolic phospholipase A₂ (cPLA₂) and/or the secreted PLA₂, are also required for the formation of eicosanoids. We have previously shown that short-term (30–120 min) exposure of monocytes to EBV primes these cells for increased release of arachidonic acid and formation of leukotrienes, a process that appeared to involve the activation of the cPLA₂, as observed by its increased phosphorylation (Ser505) and translocation (16). It is, however, distinctly possible that prolonged exposure (12–24 h) of monocytes to EBV impacts on the expression of cPLA₂ and/or secreted PLA₂ and consequently on PG biosynthesis. Studies are in progress to assess the effect of EBV on arachidonate release and PLA₂ expression under the experimental conditions used in the present studies.

Our data suggest that EBV impairs the activation of the NF-κB transcription factor. Several studies have reported the importance of NF-κB in the induction of COX-2 gene transcription in both monocytes and monocytic cell lines (34, 35). In fact, the COX-2 gene was shown to possess two NF-κB binding sites located within its promoter (36, 37). In the present study, we show that EBV decrease LPS-induced translocation of NF-κB from the cytoplasm, where it is found as an inactive complex with I-κB, to the nucleus, where it mediates transcriptional activation. Interestingly, p65, a component of the NF-κB dimer, was shown to physically interact with the EBV immediate-early protein ZEBRA, which is essential to viral replication, and inhibit its transactivating activities in B lymphocytes (38). Such interaction between ZEBRA and p65 was recently demonstrated in T lymphocytes, and it was suggested that the formation of this complex is functionally analogue to the inactive I-κB/NF-κB complex found in the cytoplasm. The occurrence of such inactive NF-κB/ZEBRA complex in EBV-infected monocytes remains to be investigated. Another possible mechanism by which EBV could inhibit translocation of NF-κB is to prevent the inducible degradation of I-κB by interfering with targeting of the ubiquitinated I-κB to the proteasome. Interestingly, the EBV nuclear Ag 1 (EBNA-1) contains a glycine-alanine repeat that is responsible for its resistance to degradation through the ubiquitin-proteasome system (39). Finally, another possible means of preventing NF-κB activation that has already been described for the African swine virus is to encode for a homologue of I-κB (40). Whether EBNA-1 or other EBV proteins possessing a similar structural module interact with I-κB remains to be established.

NF-κB is also involved in the transcriptional activation of several monocyte/macrophage genes, including those that encode proinflammatory cytokines (41). For example, TNF-α gene possesses multiple NF-κB binding sites in its promoter, which makes its expression highly induced following NF-κB translocation to the nucleus (42, 43). Regulation of TNF-α gene is of particular interest because our previous observations showed that EBV strongly inhibits the transcription of this cytokine in monocytes stimulated with either LPS or phorbol esters (29), two known inducers of NF-κB activation (44). Taken together, it is tempting to speculate that regulation of COX-2 and TNF-α genes by EBV could result from impaired NF-κB activation.

Other viruses, such as herpes simplex virus type 1 and human CMV, have been shown to up-regulate PGE₂ production in infected monocytes (45, 46). Similarly, it was reported that the formation of both 5-lipoxygenase and COX products derived from arachidonic acid was enhanced in HIV-infected monocytes (47). Thus, the effects of EBV on the arachidonate metabolism are particular, in that while the 5-lipoxygenase pathway is up-regulated,
resulting in increased leukotriene synthesis, the COX-2 pathway is inhibited. To our knowledge, this is the first report of PGE₂ bio-synthesis inhibition by human viruses. In addition, our results indicate that expression of EBV late cycle proteins is involved in such inhibition. Although the EBV-suppressive effect reported herein is likely to result from an inhibition of NF-κB activation, the nature of EBV interactions with the transductional events that trigger NF-κB activation remains to be established.

Arachidonic metabolites are important immunomodulatory mediators, and interference with the tightly regulated equilibrium between leukotriene and PG productions may represent an additional mechanism utilized by EBV to disturb the immune response and promote viral replication.

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References