Epstein-Barr Virus Modulates De Novo Protein Synthesis in Human Neutrophils

By André D. Beaulieu, Robert Paquin, and Jean Gosselin

Neutrophils and macrophages represent the first line of defense against microbial invaders. However, the role of phagocytes in host response to viral infection is poorly understood. We have previously shown that Epstein-Barr virus (EBV) interacts with human monocytes and modulates cytokine production in this cell type, but its effects on neutrophils are still unknown. In the present study, we investigated the presence of EBV receptor (CR2 or CD21) on neutrophils by cytofluorometry using five different anti-CD21 monoclonal antibodies (MoAbs), as well as fluoroscein isothiocyanate-EBV (FITC-EBV). Whereas no significant amount of neutrophils reacted with anti-CD21 MoAbs, studies with FITC-EBV indicated that viral particles bind to 30% of cells (in some individuals, EBV binds to more than 50% of neutrophils). This interaction is specific as it was completely inhibited by nonconjugated virus or with labeled virus preincubated with neutralizing MoAbs. After EBV treatment, cellular aggregation was observed in neutrophil cultures, an indication that neutrophils were activated. Although EBV did not induce respiratory burst activity in neutrophils, pretreatment with infectious particles enhanced (priming effect) the fMLP-induced O2 release in neutrophils. Instead of restricting our analysis to specific cytokine genes, we investigated the effects of EBV on neutrophil transcriptional events in general. The effect of this virus on de novo synthesis of total cellular RNA was first investigated by measuring the incorporation of [5-3H]uridine into total RNA. The results showed that RNA synthesis in neutrophils was significantly increased (2.3- to 21.3-fold) by EBV compared with the unstimulated controls. Live and UV-inactivated virus markedly induced RNA synthesis, whereas heat-inactivated virus lost this ability. Induction of RNA transcription was EBV specific, as an EBV-neutralizing antiserum abolished this effect. Induction of protein synthesis was also studied by measuring the incorporation of [35S]methionine and [35S]cysteine into secreted and intracellular proteins in neutrophils incubated with EBV. The synthesis of both secreted and cytoplasmic proteins was induced by EBV. One- and two-dimensional gel electrophoresis analysis showed that EBV modulates protein synthesis, because activation of the synthesis of certain proteins was accompanied by the inhibition of others. Interleukin-1β (IL-1 β) and IL-1 receptor antagonist (IL-1Ra) synthesis was found to be induced by EBV. Therefore, modulation of host-response proteins such as IL-1Ra could be one of the many mechanisms by which this virus avoids rejection. However, the importance of our findings in understanding neutrophil responses to EBV must be fully determined to better understand the biology of this virus in the host.

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Because EBV target tropism seems to be more widespread than believed, and because neutrophils are involved in the early events of the natural immunity, we studied the interactions of EBV with neutrophils and the effects of the virus on this cell type. Our results indicate that EBV specifically binds to human neutrophils. Preliminary studies designed to analyze the events that modulate gene expression in neutrophils showed that EBV has a marked capacity to activate the nuclear signaling events necessary in neutrophils for the initiation of de novo RNA synthesis. The results of the present study show that EBV increases the level of RNA synthesis and modulates (ie, activates or suppresses) de novo production of selective proteins in neutrophils. IL-1β and IL-1Ra synthesis were found to be induced by EBV. Because IL-1Ra is known to be a natural inhibitor of IL-1 and of DNA synthesis in mitogen-stimulated lymphocytes,15-16 our observations suggest that induction of this antagonist by EBV as well as induction of a number of other proteins could represent another mechanism used by the virus to disrupt the immune response.

MATERIALS AND METHODS

Neutrophil preparation. Neutrophils were isolated from venous blood obtained from normal healthy volunteers using Ficoll-Hypaque density centrifugation (Pharmacia Biotech Inc, Baie d'Urfe, Quebec, Canada) as previously described. All neutrophil preparations contained fewer than 1% monocytes as determined by monocytes density centrifugation and less than 0.3% of B and T lymphocytes, as evaluated by cytometry using anti-CD2, anti-CD3, and anti-CD19 monoclonal antibodies (MoAbs; Becton Dickinson, San Jose, CA). Viability, estimated by the trypan blue-exclusion procedure, was greater than 99% in all preparations.

Virus preparation. Viral preparations of EBV strain B95-8 were produced as previously described. Briefly, B95-8 cells (which were mycoplasma-free tested) were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). When the viability of cell cultures reached 20% or less, as determined by trypan blue exclusion, cell-free culture supernatants were harvested and filtered through a 0.45-μm pore size filter, and viral particles were purified by differential ultracentrifugation. Virus stocks were resuspended in RPMI-1640, aliquoted and stored at −80°C until use. Viral titers were measured as described and evaluated at 10^3 transforming units (TFU/mL).

General incubation conditions. Neutrophils were incubated in either Hanks' balanced saline solution (HBSS) or RPMI-1640 medium supplemented with 10% decomplemented autologous plasma and 1% heat-inactivated fetal bovine serum (FBS). Cell viability was determined by trypan blue-dye exclusion, cell-free culture supernatants were incubated in 96-well microtiter plates and treated with infectious EBV at 10^6 TFU/mL. The plates were incubated for 5 hours at 37°C, 5% CO_2 in a humid atmosphere, after which the cells were harvested on glass fiber filter discs, and radioactivity was measured in a liquid scintillation counter.

Neutrophil isolation and purification. Neutrophils were purified by differential centrifugation. Virus stocks were resuspended in RPMI-1640, aliquoted and stored at −80°C until use. Viral titers were measured as described and evaluated at 10^3 transforming units (TFU/mL).

Superoxide production (O2−) assay. Superoxide production was measured by performing superoxide dismutase-sensitive reduction of cytochrome C based on a method previously described elsewhere. Briefly, neutrophils (5 × 10^6 cells/mL) were suspended in HBSS buffer supplemented with 1% decomplemented autologous plasma. Then 100 μL of the cell suspensions was incubated in 96-well microtiter plates in the presence of 1 μmol/L cytochrome C for 5 minutes at 37°C. The reactions were stopped by transferring the tubes to an ice-cold bath followed by the addition of superoxide dismutase (final concentration 62 μg/mL) and centrifugation. The optical density of the supernatants was read at 550 nm.

RNA synthesis assay. The synthesis of RNA by neutrophils was studied as previously described by measuring the incorporation of [3H]-uridine into total RNA. Briefly, neutrophils (5 × 10^6 cells/mL) were suspended in HBSS buffer supplemented with 1% decomplemented autologous plasma. Then 100 μL of the cell suspensions was incubated in 96-well microtiter plates in the presence of 1 μCi/mL [3H]-uridine and treated with GM-CSF, rIL-1, or infectious EBV (10^6 TFU/mL). The plates were incubated for 5 hours at 37°C, 5% CO_2 in a humid atmosphere, after which the cells were harvested on glass fiber filter discs, and radioactivity was measured in a liquid scintillation counter.

Metabolic labeling of neutrophils. Neutrophils were cultured in the presence of [3H]-uridine to total RNA. Briefly, neutrophils (5 × 10^6 cells/mL) were suspended in HBSS buffer supplemented with 1% decomplemented autologous plasma. Then 100 μL of the cell suspensions was incubated in 96-well microtiter plates in the presence of 1 μCi/mL [3H]-uridine and 1% heat-inactivated fetal bovine serum (FBS). Cell viability was determined by trypan blue exclusion, cell-free culture supernatants were harvested and filtered through a 0.45-μm pore size filter. Viral particles were purified by differential ultracentrifugation. Virus stocks were resuspended in RPMI-1640, aliquoted and stored at −80°C until use. Viral titers were measured as described and evaluated at 10^3 transforming units (TFU/mL).

General incubation conditions. Neutrophils were incubated in either Hanks' balanced saline solution (HBSS) or RPMI-1640 medium supplemented with 1% decomplemented autologous plasma and 1% decomplemented autologous plasma. Neutrophils were incubated at a density of 10^6/mL in methionine- and cysteine-free RPMI 1640. Labeling was performed in the presence of 1% decomplemented FBS with 100 μCi/mL [3H]-methionine (1,000 Ci/mmol) and [3S] cysteine (1,300 Ci/mmol) at a concentration of 125 μCi/mL for each label and treated with infectious EBV (10^6 TFU/mL) or with GM-CSF (3 nmol/L). Cellular suspensions were incubated for 3 hours at 37°C, 5% CO_2 in a humid atmosphere. The cells were then centrifuged at 400g for 10 minutes, and the cell incubation media as well as the cell lysates were collected in the presence of protease inhibitors (aprotinin, 60 trypsin inhibiting units/mL, phenylmethylsulfonyl fluoride, 2 μmol/mL). Labeled proteins were precipitated with 70% ethanol and harvested onto a filter, which was placed in scintillation vials with 4 mL of Aquasol-2 (DuPont NEN, Dorval, Quebec, Canada) for counting.

Immunosolation of IL-1Ra. Cell culture supernatants from unstimulated, EBV−, and GM-CSF-treated neutrophils were performed for immunoisolation of IL-1Ra as previously described by using a monoclonal rabbit antibody to IL-1Ra, a gift from Dr William Arend (University of Colorado School of Medicine, Denver).

RNA isolation and Northern blotting. RNA isolation and Northern blotting were performed as previously described. Harvested RNA was electrophoresed on a 1% agarose gel, transferred to a nylon membrane, and hybridized with a 32P-labeled probe corresponding to the site of the PSP64 plasmid. The blots were hybridized with the probe and washed at high stringency at 65°C in 0.1× sodium chloride/sodium phosphate/SDS (SSC) for 2 hours. The blots were then washed in 2× SSC at 65°C for 30 minutes and hybridized with a 32P-labeled probe corresponding to the site of the PSP64 plasmid. The blots were hybridized with the probe and washed at high stringency at 65°C in 0.1× sodium chloride/sodium phosphate/SDS (SSC) for 2 hours.

RNA assay for EBV receptor expression. Assay for EBV receptor expression. Freshly isolated neutrophils (1 × 10^6/mL) were stained with different anti-CR2 MoAbs as described by the suppliers. The following MoAbs were used: OKB7 (Ortho Diagnostic Systems, Raritan, NJ), B2 (Coulter Immunology, Hialeah, FL), H5B (Becton Dickinson, San Jose, CA), B-E5 (Sero-toc, Toronto, Ontario, Canada), and 10B1a (Amac, Mississauga, Ontario, Canada). For EBV binding, 50 μL of FITC-EBV was added to a pellet of 1 × 10^6 cells. The mixture was incubated for 60 minutes in the dark at 4°C with frequent agitation. The cells were then washed three times with cold phosphate-buffered saline (PBS) at pH 7.2, resuspended in 0.5 mL of 0.5% paraformaldehyde in PBS, and analyzed by flow cytometry using an EPIC 753 (Coulter, Miami Lakes, FL). Raji cell lines were used as positive controls. The percentage of positive cells was determined from samples of 10,000 cells.

One- and two-dimensional gel electrophoresis. For one-dimen-
sional (1-D) gel electrophoresis, samples consisted of supernatants from the metabolically labeled neutrophils. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Hoefer slab gel electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, CA), according to the method described by Laemmli.\(^3^4\) Samples for two-dimensional (2-D) gel electrophoresis consisted of cell lysates prepared as previously described.\(^3\) First-dimension isoelectric focusing was performed using 2% ampholite and 2-D gels were 12% acrylamide. The 1-D and 2-D gels were processed for fluorography by washing in water for 15 minutes and treatment with 1 mol/L sodium salicylate, pH 7.0, for 15 minutes, dried, and exposed to Kodak XAR-5 films (Eastman Kodak, Rochester, NY) at \(-70^\circ\text{C}\).

RESULTS

Analysis of CR2 expression on human neutrophils. The presence of EBV receptor (CR2 or CD21 antigen) expressed on the membrane surface of neutrophils was investigated using five anti-CR2 MoAbs that detect distinct epitopes across the molecule: OKB7, B2, HB5, B-E5, and 10B1a. Analysis of positive cells was performed by flow cytometry. In all cases, freshly isolated neutrophils were either clearly negative (Fig 1A, B, and D) or weakly expressed the CR2 antigen (Fig 1C, 5% and 1E, 2%) when compared with Raji cells (Fig 1F, 98%).

**EBV binding to human neutrophils.** Although the presence of CR2 antigen was not significantly detected on neutrophils, we investigated the potential of EBV to specifically bind to human neutrophils. We also studied the ability of EBV to induce cellular aggregation, as an indication of neutrophil activation.

We first labeled purified EBV particles with FITC and incubated such viral particles (FITC-EBV) with neutrophils. Analysis by flow cytometry showed that a significant proportion of cells was positive (Fig 1H, 30%) (in some donors, FITC-EBV bound to more than 50% of neutrophils). We confirmed the specificity of EBV binding by pretreating viral particles with MoAb 72A1; this antibody reacts with the gp350 of the viral envelope and blocks the binding of the virus to its receptor. Pretreatment of FITC-EBV with 72A1 totally inhibited the binding of the virus to neutrophils (Fig 1G). As expected, preincubation of neutrophils with anti-CR2 MoAbs did not affect EBV binding. Furthermore, interaction of EBV with neutrophils resulted in cellular aggregation. In fact, as early as 5 hours post-EBV treatment, neutrophil aggregation was observed (Fig 2B). Again, specificity of EBV effect was confirmed by pretreating viral particles with anti-serum containing neutralizing antibodies. Such a treatment markedly abolished cellular aggregation induced by EBV (Fig 2C).

Effect of EBV on respiratory burst response. To eliminate invading microorganisms such as bacteria or viruses, neutrophils use microbicidal processes related to the formation of reactive oxygen, the best known being the respiratory burst activity. Therefore, the effects of EBV on this microbicidal activity were evaluated. The results obtained showed that EBV did not significantly induce the production of O\(_2^-\) in neutrophils (Table 1). However, EBV was found to prime human neutrophils in the induction of superoxide release by FMLP.

**Induction of de novo RNA synthesis by EBV.** We incubated human peripheral blood neutrophils in the presence of EBV and [5-\(^3\)H] uridine to measure the incorporation of this label into de novo synthesized RNA. The data obtained as a result of studying 10 different donors are presented in Table 2. Incorporation of [5-\(^3\)H] uridine was maximal at 5 hours (data not shown). Cells incubated with GM-CSF or FMLP were always included in the experiments as positive controls. The viability of neutrophils, as determined by trypan blue-dye exclusion, was greater than 96% after 5 hours in all experimental conditions. In all cases, EBV induced a

![Fig 1. Cytofluorometric analysis of EBV receptor on human neutrophils. Neutrophils were stained with different anti-CR2 MoAbs or with FITC-conjugated EBV. (A) Anti-CR2 OKB7. (B) Anti-CR2 10B1a. (C) Anti-CR2 B-E5. (D) Anti-CR2 B2. (E) Anti-CR2 HB5. (F) Raji cells stained with anti-CR2 B-E5 as positive control. Raji cells were also tested with all anti-CR2 MoAbs and were positive in each case. (G) Neutrophils were treated with neutralized FITC-EBV using MoAbs 72A1. (H) Binding of FITC-EBV on neutrophils. Dark surfaces represent negative control evaluated with isotype-matched antibody or FITC alone. Each histogram is representative of three experiments. Fluorescence is expressed on a logarithmic scale.](www.bloodjournal.org)
Fig 2. Effect of EBV on neutrophil aggregation. Neutrophils (1 x 10^6 cells/mL) were treated with infectious EBV during 5 hours and cellular aggregation was visualized with an inverted microscope. (A) Unstimulated cells. (B) EBV-treated cells. (C) Neutrophils treated with viral particles neutralized with an immune serum (original magnification x 100). This was observed in 10 other experiments.
EBV INTERACTS WITH HUMAN NEUTROPHILS

Table 1. Effect of EBV on O2 Generation by Human Neutrophils

<table>
<thead>
<tr>
<th>Donor</th>
<th>Nonstimulated</th>
<th>EBV</th>
<th>fMLP</th>
<th>EBV + fMLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>0.00</td>
<td>11.32± 1.61</td>
<td>20.15± 2.31*</td>
</tr>
<tr>
<td>2</td>
<td>0.62 ± 0.55</td>
<td>1.09 ± 0.68</td>
<td>9.38 ± 2.13</td>
<td>18.47 ± 0.95*</td>
</tr>
<tr>
<td>3</td>
<td>0.18 ± 0.16</td>
<td>0.38 ± 0.33</td>
<td>9.86 ± 0.66</td>
<td>17.24 ± 0.84*</td>
</tr>
<tr>
<td>4</td>
<td>0.15 ± 0.14</td>
<td>1.53 ± 1.09</td>
<td>9.03 ± 0.06</td>
<td>10.08 ± 0.86*</td>
</tr>
</tbody>
</table>

Neutrophils (2 × 10^6 cells/mL) from four healthy donors were treated with EBV (45 minutes at 37°C), with fMLP (1 μmol/L) (10 minutes at 37°C) alone, or in combination with both agonists. Superoxide release was measured as described in Materials and Methods. Results are expressed in nmol/mL of O2 ± SE.

* Data significantly different from fMLP-treated cell controls (P < .05) using unpaired two-tailed Student’s t-test.

marked uptake of [5-3H] uridine, indicating that RNA synthesis had been initiated after activation of neutrophils by the virus. The incorporation of the label was reduced to or below background levels in the presence of actinomycin D, an inhibitor of transcription (data not shown). Stimulation indices were calculated by dividing the number of counts obtained with the activated cells by that obtained with nonactivated neutrophils. With EBV-stimulated cells, these indices varied between 2.3 and 21.3 among donors and closely paralleled the indices obtained with GM-CSF– or fMLP–activated neutrophils.

**Induction of de novo protein synthesis by EBV.** To determine the effects of EBV on de novo protein synthesis, we incubated neutrophils with EBV for 20 hours (the time we found to be optimal; data not shown) in the presence of [35S] methionine and [3H] cysteine. Cell supernatants and cell lysates were precipitated with 70% ethanol to quantitate synthesized proteins. Results are presented in Table 3, and show that EBV induced de novo synthesis of extracellular and intracellular proteins by neutrophils to a degree that generally paralleled the stimulation by GM-CSF. In an attempt to demonstrate that the induction of RNA and de novo protein synthesis is a direct effect of EBV, we performed a number of additional experiments. Neutrophils from six healthy donors were stimulated with infectious or inactivated EBV. RNA synthesis was evaluated in three donors and extracellular protein synthesis was procured in three others. In all cases, the effect of EBV was suppressed when inactivated virus was used (Table 4). However, in contrast to heat-inactivated virus, UV-irradiated EBV retained the full capacity of live virus to induce RNA or protein synthesis in neutrophils. We also confirmed the specificity of EBV by pretreating viral particles with neutralizing antibodies. These results suggest that the observed effects are related to EBV and that binding of the native particles is sufficient to induce such an effect.

**Table 2. Effect of EBV on RNA Synthesis in Human Neutrophils**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Nonstimulated</th>
<th>EBV</th>
<th>GM-CSF</th>
<th>fMLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>649 ± 30</td>
<td>2,282 ± 246 (3.5)*</td>
<td>2,100 ± 84 (3.2)</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>307 ± 100</td>
<td>6,536 ± 760 (21.3)</td>
<td>15,550 ± 777 (50.7)</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>460 ± 70</td>
<td>6,520 ± 904 (14.2)</td>
<td>5,380 ± 470 (11.7)</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>626 ± 207</td>
<td>2,212 ± 151 (3.5)</td>
<td>2,267 ± 231 (4.1)</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>609 ± 125</td>
<td>2,148 ± 163 (3.5)</td>
<td>ND</td>
<td>2,296 ± 62 (3.8)</td>
</tr>
<tr>
<td>6</td>
<td>753 ± 101</td>
<td>1,736 ± 214 (2.3)</td>
<td>ND</td>
<td>3,986 ± 51 (6.3)</td>
</tr>
<tr>
<td>7</td>
<td>729 ± 59</td>
<td>1,865 ± 141 (2.6)</td>
<td>ND</td>
<td>1,587 ± 64 (2.2)</td>
</tr>
<tr>
<td>8</td>
<td>475 ± 272</td>
<td>1,808 ± 39 (3.8)</td>
<td>ND</td>
<td>1,324 ± 140 (2.8)</td>
</tr>
<tr>
<td>9</td>
<td>3,324 ± 158</td>
<td>11,977 ± 104 (3.6)</td>
<td>ND</td>
<td>5,601 ± 387 (1.7)</td>
</tr>
<tr>
<td>10</td>
<td>2,496 ± 389</td>
<td>7,779 ± 171 (3.1)</td>
<td>ND</td>
<td>5,524 ± 414 (2.3)</td>
</tr>
</tbody>
</table>

Neutrophils were cultured in Hanks’ balanced salt solution containing [5-3H] uridine and stimulated with infectious EBV, GM-CSF (3 mmol/L), or fMLP (10−10 mol/L) for 5 hours. RNA synthesis was measured as described in Materials and Methods. Results, expressed in cpm, are the means of quadruplicate ± SE. Nonspecific labeling of viral particles with [5-3H] uridine was always below 180 ± 29 cpm.

**Abbreviation:** ND, not done.

* Numbers in parentheses represent the index of stimulation.
plasmic protein synthesis induced by EBV or GM-CSF, we compared from unstimulated, GM-CSF- and EBV-activated neutrophils. The major proteins (or group of proteins) of neutrophils were identified by the letters b, c, f, g, i, and j are inhibited by EBV, whereas those identified by the letters a, d, e, h, and k (IL-1Ra) are stimulated.

Two-dimensional gel analysis of intracellular proteins induced by EBV. To obtain better resolution of total cytoplasmic protein synthesis induced by EBV or GM-CSF, we performed 2-D gel analysis. Fluorograms of [35S] methionine- and [35S] cysteine-labeled products are shown in Fig 5A (unstimulated cells) and Fig 5B (EBV-activated cells). The de novo synthesis of a number of intracellular proteins was strongly affected by EBV, with some activated and other proteins inhibited. The major proteins (or group of proteins) of interest are identified by letters. Proteins identified by the letters a, d, e, h, and j are inhibited by EBV, whereas those identified by the letters b, c, f, g, i, and k (IL-1Ra) are stimulated.

### Table 3. Protein Synthesis Induced by EBV in Human Neutrophils

<table>
<thead>
<tr>
<th>Donor</th>
<th>Nonstimulated</th>
<th>EBV</th>
<th>GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exocellular proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>540,837 ± 27,041</td>
<td>2,027,208 ± 283,537</td>
<td>695,439 ± 13,908</td>
</tr>
<tr>
<td>2</td>
<td>567,465 ± 56,766</td>
<td>696,293 ± 13,965</td>
<td>897,129 ± 143,541</td>
</tr>
<tr>
<td>3</td>
<td>281,620 ± 50,691</td>
<td>594,300 ± 58,860</td>
<td>307,140 ± 3,072</td>
</tr>
<tr>
<td>4</td>
<td>329,662 ± 13,186</td>
<td>595,853 ± 65,543</td>
<td>387,936 ± 8,688</td>
</tr>
<tr>
<td>5</td>
<td>306,246 ± 40,071</td>
<td>489,021 ± 19,560</td>
<td>349,327 ± 6,868</td>
</tr>
<tr>
<td>Intracellular proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>765,910 ± 183,818</td>
<td>1,656,852 ± 196,822</td>
<td>1,014,954 ± 111,644</td>
</tr>
<tr>
<td>2</td>
<td>335,792 ± 106,863</td>
<td>1,495,162 ± 164,907</td>
<td>1,459,792 ± 29,195</td>
</tr>
<tr>
<td>3</td>
<td>372,833 ± 74,566</td>
<td>1,491,576 ± 59,683</td>
<td>986,579 ± 164,318</td>
</tr>
<tr>
<td>4</td>
<td>497,260 ± 9,945</td>
<td>765,944 ± 11,485</td>
<td>748,319 ± 52,242</td>
</tr>
<tr>
<td>5</td>
<td>176,334 ± 86,106</td>
<td>1,083,931 ± 182,589</td>
<td>1,539,664 ± 230,949</td>
</tr>
</tbody>
</table>

Cells were cultured in RPMI-1640 containing [35S] methionine and [35S] cysteine and treated with infectious EBV, or GM-CSF (3 nmol/L) for 20 hours. Exocellular and intracellular protein fractions were obtained and analyzed as described in Materials and Methods. Results are the mean (cpm) of quadruplicate ± SE, in all samples, viability, estimated by the trypan blue-dye exclusion procedure, was greater than 90%.

was capable of inducing de novo synthesis of IL-1Ra by neutrophils to a degree similar to GM-CSF. The presence of IL-1Ra and IL-1β mRNA transcripts was also evaluated. Figure 4B shows Northern blots performed with RNA prepared from unstimulated, GM-CSF- and EBV-activated neutrophils. The results demonstrate that the synthesis of IL-1Ra and IL-1β transcripts is induced by GM-CSF (lane 2) as well as by EBV (lane 3) compared with unstimulated cells (lane 1). GAPDH RNA was used as internal control.

DISCUSSION

In the EBV system, little is known about the interactions of EBV with phagocytes, such as monocytes and neutrophils, despite the fact that these cells are believed to constitute the key elements in the nonspecific defense mechanisms against viral infection. Furthermore, viral invasion induces in the host an inflammatory response in which neutrophils and monocytes are likely to play central roles. To date, information on virus–neutrophil interactions has come mainly from studies performed with the influenza A virus,28 which cannot only bind to the surface of neutrophils and activate these cells, but also render them dysfunctional.22,23

Until recently, EBV target cell tropism was believed to be limited to epithelial cells of the oropharynx as well as to B lymphocytes. Growing evidence suggests that T lymphocytes may also be subjected to EBV targeting in vivo.24 Furthermore, interaction of this virus with monocytes has also been clearly shown.11,12 Because binding of EBV to the monocyte’s surface was accompanied by alterations in the level of cytokine synthesis in this cell type, strongly suggesting that this virus can modulate gene expression in monocytes, we hypothesized that EBV exerts a similar effect

### Table 4. Effects of Infectious and Inactivated EBV on RNA and Protein Synthesis by Human Neutrophils

<table>
<thead>
<tr>
<th>Donor</th>
<th>Nonstimulated</th>
<th>EBV</th>
<th>UV-irradiated EBV</th>
<th>Heat-Inactivated EBV</th>
<th>Neutralizing Antibodies + EBV</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA synthesis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>422 ± 88</td>
<td>3,309 ± 165</td>
<td>3,472 ± 1,180</td>
<td>601 ± 44*</td>
<td>690 ± 34*</td>
</tr>
<tr>
<td>2</td>
<td>312 ± 102</td>
<td>2,068 ± 450</td>
<td>2,162 ± 170</td>
<td>848 ± 104*</td>
<td>444 ± 52*</td>
</tr>
<tr>
<td>3</td>
<td>220 ± 10</td>
<td>2,239 ± 524</td>
<td>2,364 ± 286</td>
<td>322 ± 34*</td>
<td>229 ± 19*</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>137,463 ± 4,020</td>
<td>283,789 ± 2,837</td>
<td>302,995 ± 10,339</td>
<td>172,060 ± 11,300*</td>
<td>161,941 ± 21,832*</td>
</tr>
<tr>
<td>5</td>
<td>450,863 ± 67,629</td>
<td>957,525 ± 134,053</td>
<td>1,121,061 ± 154,529</td>
<td>342,898 ± 68,577*</td>
<td>568,945 ± 79,652*</td>
</tr>
<tr>
<td>6</td>
<td>286,269 ± 5,725</td>
<td>1,306,843 ± 196,026</td>
<td>1,290,678 ± 119,415</td>
<td>440,810 ± 97,321*</td>
<td>559,544 ± 50,368*</td>
</tr>
</tbody>
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Cells from six different donors were stimulated with infectious viral particles or with UV-irradiated (45 minutes, 265 nm) or with heat-inactivated (1 hour at 56°C) EBV for 20 hours. Immune serum was used to neutralize live virus. In appropriate samples where cells were treated with actinomycin D (5 μg/mL) or with cycloheximide (5 μg/mL) before EBV stimulation, results were below those of nonstimulated cell controls. RNA and extracellular protein synthesis were measured as described in Materials and Methods. Results are the mean (cpm) of triplicate ± SE.

* Values significantly different (P < .05) from respective EBV-treated controls, using unpaired two-tailed Student’s t-test.
on neutrophils. We first investigated the presence of EBV receptor (CD21) on the membrane surface of neutrophils using five anti-CD21 MoAbs that recognize distinct epitopes across the molecule. We did not detect significant expression of this antigen on neutrophils. At most, 5% of cells were found to be positive with one MoAb (B-E5). However, when using labeled viral particles, more than 30% and, in some cases, 50% of the cells bound to EBV. We then considered the possibility that EBV binds to a molecule that is distinct from CD21. This is in perfect agreement with other studies that showed the presence of C3d receptor (also known as the EBV receptor) on neutrophils and monocytes and the specific adsorption of EBV to monocytes/macrophages independently of CD21. Similarly, the binding of EBV particles was also demonstrated in studies with HSB-2 T-cell lines, human thymocytes, and monocytic cell lines, even if the reactivity of MoAbs to CD21 was negative. These findings could suggest the existence of an EBV receptor that exhibits phenotype characters distinct from the CD21 antigen, and our results support the hypothesis of the presence of another EBV receptor on the surface membrane of neutrophils as proposed in other systems.

Fig 3. De novo protein synthesis by human neutrophils. Cells were labeled with [35S]methionine and [35S]cysteine in the presence of 0.1% FBS for 20 hours. Proteins secreted in supernatants were isolated and separated by SDS-PAGE as described in Materials and Methods. (A) Nonstimulated cells. (B) GM-CSF–treated cells. (C) EBV–treated cells. Arrow indicates protein of an approximate molecular weight of 23 kD (IL-1Ra). In all samples, viability was greater than 90% as estimated by trypan blue-dye exclusion. Results presented here were reproducible with three other donors.

Fig 4. Induction of IL-1Ra and IL-1β in EBV-treated neutrophils. Cells were incubated for 5 hours before RNA isolation or 20 hours before procuring supernatants. (A) Immunoblotting of IL-1Ra protein using a polyclonal rabbit antibody to IL-1Ra. Lane 1, nonstimulated cells; lane 2, GM-CSF–treated cells; lane 3, EBV–treated cells. (B) Northern blot analysis of IL-1RA and IL-1β mRNA transcripts. B1, unstimulated cells; B2, GM-CSF–treated cells; B3, EBV–treated cells. Results are representative of four different experiments.
Fig 5. Fluorograms from 2-D gel electrophoresis showing de novo protein synthesis in EBV-treated cells. Cells were labeled with [35S]methionine and [35S]cysteine in the presence of 0.1% FCS for 20 hours. Intracellular proteins from unstimulated and stimulated cells were submitted to 2-D gel electrophoresis followed by fluorography. (A) Nonstimulated cells. (B) EBV-treated cells. Letters indicate proteins or group of proteins modulated by EBV as compared with unstimulated cell controls. In all samples, viability, estimated by trypan blue-dye exclusion, was greater than 90%. Results presented are representative of four different donors.

After incubation of neutrophils with EBV, it was of particular interest to note that marked aggregation of neutrophils was observed after 4 to 5 hours of incubation, an indication that the cells had been activated. No aggregation was observed in the control cells during the whole procedure. Furthermore, in contrast to other microorganisms, EBV does not itself induce a respiratory burst activity in neutrophils, but instead enhances their potential for $O_2^*$ production when subsequently stimulated with fMLP. The fact that neutrophils are primed or are more sensitive to $O_2^*$ release after EBV treatment, may represent an alternative pathway for these cells to neutralize viral invasion.

Instead of focusing our attention on specific genes, as was the case for IL-6 and TNF-α in monocytes, we investigated the effects of EBV on transcriptional events in general. We used an assay that allows the quantitative assessment of de novo RNA synthesis by measuring the level of incorporation of [5-3H]uridine into total RNA. The level of neutrophil RNA synthesis clearly increased when these cells were incubated with EBV. Although induction of RNA synthesis varied quantitatively between individuals, it closely paralleled that induced by either GM-CSF or fMLP, indicating individual susceptibility to activation by EBV rather than experimental variation.

Although RNA synthesis was detected in EBV-activated neutrophils, the possibility existed that only ribosomal RNA was being synthesized. To eliminate this possibility, we studied whether the newly transcribed RNA was translated into proteins. We incubated EBV-treated neutrophils in the presence of [35S]methionine and [35S]cysteine and compared the amount of proteins obtained with untreated and EBV-treated neutrophils. Based on the results, it was clear that the EBV-treated cells synthesized proteins to an extent significantly greater than the nontreated cells. The synthesis of both intracellular and extracellular proteins was increased, and, as for RNA synthesis, variations between individuals paralleled those observed with GM-CSF.

In light of the above observations, it became important to show that the effects on the modulation of gene expression in neutrophils were EBV specific. To learn more about the ability of EBV to induce initial signals leading to the synthesis of proteins by neutrophils, we studied the effects of UV- and heat-inactivated virus on RNA and extracellular protein synthesis. The present study shows that live as well as UV-irradiated EBV can upregulate RNA and protein synthesis, suggesting that contact with a structural protein is sufficient to induce such an effect and that RNA produced in neutrophils is not associated to viral RNA.

Because we had confirmed the specificity of EBV effects in terms of protein synthesis in neutrophils, we next extended our study on the synthesis of intracellular proteins by using 2-D gel analysis. Comparison of fluorograms from unstimulated and EBV-treated neutrophils showed that the synthesis of a number of proteins was induced, whereas others were inhibited. Such results suggest that EBV can modulate gene expression in neutrophils (ie, activation or suppression) as reported in the monocytic cell population. Among the number of proteins induced by EBV, we have identified a protein with a molecular weight of approximately 23 kD. By using immunoblot analysis, this protein was found to be IL-1Ra, a molecule produced by a variety of tissue macrophages as well as by blood monocytes and neutrophils. In addition, IL-1β synthesis was also found to be induced by
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EBV. In vivo, the production of this inflammatory cytokine is characterized by an increase in the number of circulating neutrophils, as well as lymphocytes to eliminate the spread of the invading agent. On the other hand, production of IL-1Ra may contribute to limiting the severity of infection, but may be inadequate in acute synthesis. IL-1Ra is known to be a natural inhibitor of many biologic functions, including actions of IL-1β,25-31 and its production by neutrophils after EBV treatment could therefore represent a mechanism used by the virus to evade, at least in part, the immune system. Alteration of biologic functions of neutrophils by such a molecule could facilitate infection of B lymphocytes, the main target of EBV. However, interaction of EBV with human neutrophils also results in the activation of other cytokine genes involved in the control of the viral infection (Roberge CJ, Gosselin J: manuscripts in preparation).

In conclusion, we found that EBV can modulate gene expression in neutrophils as reflected by the activation or suppression of the synthesis of individual proteins. Further studies are needed to understand more completely the effects of EBV on neutrophils at the early stages of the infectious process.

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