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Research article

Epstein-Barr virus reactivation after superinfection of the BJAB-B1 and P3HR-1 cell lines with cytomegalovirus

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Abstract

Background: Studies examining herpesvirus-herpesvirus (cytomegalovirus (CMV)-Epstein-Barr virus (EBV)) interactions are limited, and many of the studies have been clinical observations suggesting such an interaction exists. This report aims to examine the *in vitro* susceptibilities of BJAB-B1 and P3HR-1 cells (EBV positive Burkitt's lymphoma B-cell lines) to a CMV superinfection; and show that EBV reactivation occurs after CMV superinfects these cell lines.

Results: The BJAB-B1 and P3HR-1 cells were observed to be susceptible to a CMV superinfection by the detection of the major immediate early (MIE) viral transcript and protein (p52) expression. The BZLF1 transcript was observed in both cell lines superinfected with CMV, indicating EBV reactivation. BZLF1 protein was observed in the BJAB-B1 cells. Antigen detection was not performed in the P3HR-1 cells.

Conclusion: The results from the *in vitro* superinfections support the *in vivo* studies suggesting a CMV infection is related to an EBV reactivation and suggests that CMV may be important as a co-factor in EBV pathogenesis in the immunocompromised patient.

Background

Cytomegalovirus (CMV) and Epstein-Barr virus (EBV), in immunocompetent individuals, are etiologically associated with infectious mononucleosis as well as other disease presentations and malignancies [1,2]. Once a person has recovered from an active herpesvirus infection, it will establish a latent infection. Maintenance of viral latency is primarily determined by the immune status of the host; but there are other factors that can contribute to herpesviral reactivation [3–7].

There are numerous studies observing CMV and EBV reactivation in immunosuppressed patients. CMV is the most important pathogen among these patients and the virus can cause both direct and indirect effects [8–13]. Among solid organ transplant recipients, the effects of CMV disease are similar among all patients, with the exception being the effect on the transplanted organ [10,14–17]. The most common disease presentation of CMV in AIDS patients is retinitis [8,11]. The significance of CMV disease in transplant and HIV infected individuals goes much further than the direct pathological effects because CMV has been shown to be an immune modulator and may contribute significantly to the net immunosuppressive status of the patient [11]. This immunomodulation would

therefore increase the susceptibility to superinfections from other pathogens.

Oral hairy leukoplakia, caused by EBV, can be present in 25% of HIV infected individuals [8,18]. Other clinical presentations have been observed as well [8,19,20]. Birx et al. observed that patients with AIDS or AIDS-related disorders have a defect in the regulation of EBV infected B-cells and these patients have high circulating numbers of these infected cells [21]. Post-transplant lymphoproliferative disorder (PTLD) results from the uncontrolled lymphoproliferation of EBV infected B-cells in transplant patients [22,23]. There are many known factors for the development of PTLD, among which is the presence of an active CMV infection [13,19,22–24].

Many studies have focused upon the presence of a single herpesvirus in the transplant or AIDS patient [8–10]. There are fewer studies examining virus-virus interactions and the effect they may have on each other. Although several investigators have focused upon the relationship of human herpesvirus-6 (HHV-6) (same herpesvirus subfamily as CMV) and HIV [25–29], studies dealing with potential herpesvirus-herpesvirus interactions are very limited. Such studies would be interesting since CMV infection has been suggested as a risk factor for the development of PTLD [22,24]. Investigators have showed that a large percentage of transplant patients who had developed PTLD also had CMV disease [22,30]. In addition to CMV being implicated as a factor for the development of PTLD, serological studies have shown that patients with an active CMV infection experienced a serological profile of EBV reactivation [22,31,32]. Cross reactivities between the two viruses were ruled out as a cause for the observed immunoreactivations.

One study performed *in vitro* superinfections of EBV positive cell lines (Akata, Raji, and P3HR-1) with HHV-6. EBV reactivation was observed to occur in those cell lines that were superinfected with HHV-6 and the authors hypothesized that there was a direct effect of HHV-6 on EBV reactivation [33]. A more recent study by Vieira et al. was able to show human herpesvirus-8 (HHV-8) activation of lytic replication by CMV [34]. HHV-8 is in the same herpesvirus subfamily as EBV. The clinical studies do suggest that there may be a relationship between CMV infection and EBV reactivation, and the *in vitro* studies strengthen the notion of potential herpesvirus-herpesvirus interactions occurring. However, the effect CMV and EBV may have on each other has not specifically been investigated. Therefore this study examined an *in vitro* CMV superinfection of EBV latently infected cell lines (BJAB-B1 and P3HR-1 cells) and the effect on EBV reactivation. These B-cell lines were observed to be susceptible to a CMV superinfection and EBV reactivation occurs only in the cell lines superinfected with CMV. The observations in this study support the previous *in vivo* and *in vitro* findings by showing that potential interactions exist between CMV and EBV. Overall, these data suggest CMV may be a cofactor in EBV pathogenesis in the immunosuppressed patient.

Results

CMV transcript in BJAB-B1 and P3HR-1 cells

The CMV MIE transcript was present in the BJAB-B1 and P3HR-1 cells superinfected with CMV at all time points as shown by the real-time PCR data (Figures 1A and 2A, respectively). CMV copy standards were run for calculation of the standard curve. The CMV standards (5×10^5, 5×10^3, and 5×10^1) for the BJAB-B1 cells are shown (Figure 1C) and the standards for the P3HR-1 cells were not shown. Agarose gel analysis was run only with the BJAB-B1 cells (Figure 1D) and the greatest amount of transcript was observed at 24 hours post CMV superinfection with a decline in the latter time points. BJAB-B1 and P3HR-1 cells that were exposed to UV-irradiated CMV had no presence of the CMV MIE transcript, indicating that the virus was inactivated (Figures 1B and 2B, respectively). The uninfected cell lines were negative for the CMV MIE transcript and show that the CMV primers do not cross-react with EBV (data not shown). Table 1 (BJAB-B1) and Table 2 (P3HR-1) show the quantitative analyses calculated from the standard curve. The calculated starting quantity (SQ) values given in both tables suggest that these cell lines, although they are susceptible to CMV, do not show a high amount of infection. Mock RT-PCRs were performed in which PCR grade water was substituted for the RT enzyme. These mock reactions served as a check for DNA contamination and the mock RT-PCRs of the respective experimental groups were negative (data not shown).

CMV antigen in P3HR-1 and BJAB-B1 cells

Immunofluorescent staining for the p52 antigen was performed to further show the susceptibilities of CMV in the BJAB-B1 and P3HR-1 cell lines. The p52 gene expresses a DNA binding protein that is present in the nuclei of infected cells beginning in the early phase of infection through to the late phase. All time points for both cell lines (BJAB-B1 and P3HR-1) were positive for p52 antigen and a representative slide is shown at 24 hours post CMV superinfection (Figure 3A and 4A, respectively). All BJAB-B1 and P3HR-1 cells exposed to UV-irradiated CMV were consistently negative for the p52 antigen indicating sufficient CMV inactivation (Figures 3B and 4B, respectively). The uninfected cells from both cell lines were also negative showing no cross-reactivities with CMV (data not shown). Flow cytometric analysis was performed only with the BJAB-B1 cells. Data from the flow experiments were able to give a quantitative analysis by analyzing the fluorescence of the individual cells. Positive cells were determined by subtracting the background fluorescence of

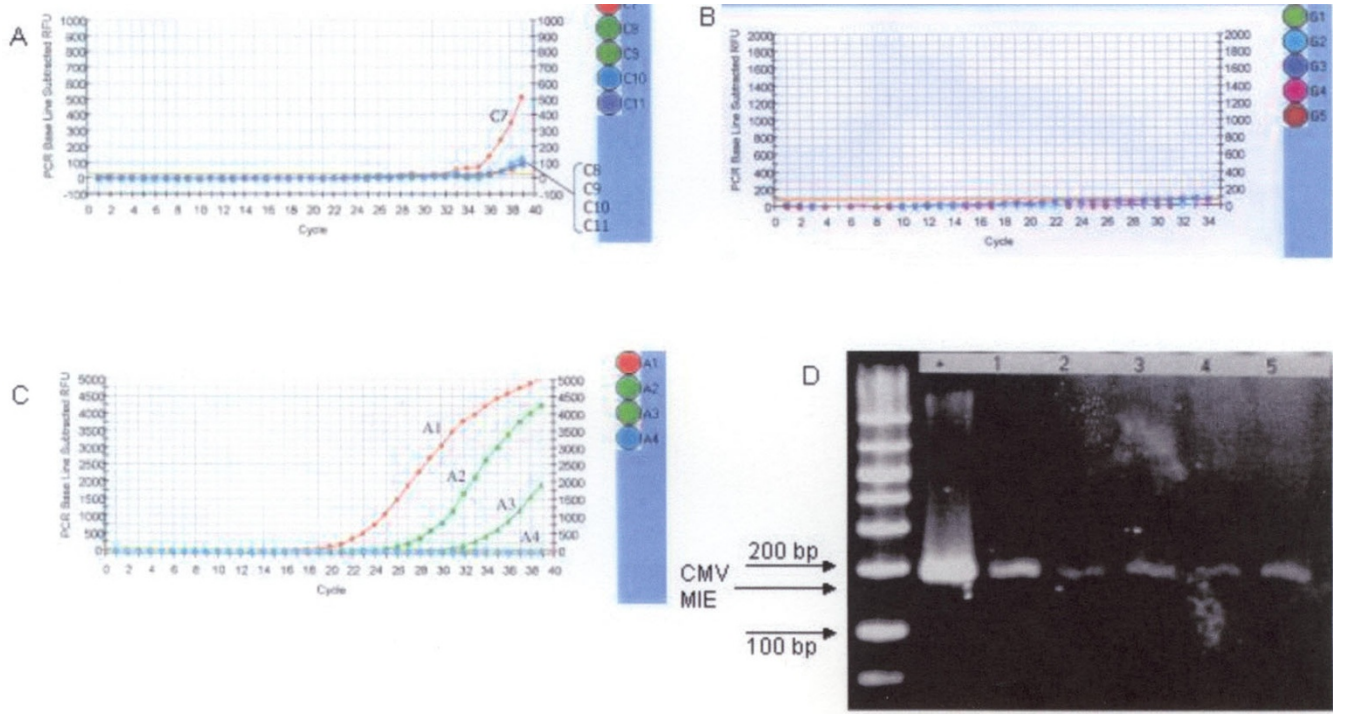

Figure 1

Real-time PCR of the CMV MIE transcript in BJAB-B1 cells. In all graphs, the axes are relative fluorescence units (RFUs) versus cycle number. Real-time PCR amplification of the CMV MIE transcript from superinfected BJAB-B1 cells was performed using 5 μl of cDNA from the RT reactions. Time points are at the given designations C7 (24 hours), C8 (48 hours), C9 (72 hours), C10 (96 hours), and C11 (168 hours) (A). Real-time PCR amplification of the CMV MIE transcript from BJAB-B1 cells incubated with UV-irradiated CMV (B). All time points were negative at the given designations G1 (24 hours), G2 (48 hours), G3 (72 hours), G4 (96 hours), and G5 (168 hours). CMV DNA copy standards were run in order to establish a standard curve for quantitative analysis. A1 (500,000 copies), A2 (5,000 copies), A3 (50 copies), and A4 (negative control) (C). Agarose (2%) gel electrophoresis of the real-time PCR samples was performed in order to show specificity of the PCR reactions. Positive control is as indicated. Shown here are the real-time PCR samples of the BJAB-B1 cells superinfected with CMV. Lanes 1–5 correlate to the time points 24, 48, 72, 96, and 168 hours (D).

the uninfected samples from either the superinfected cells or cells exposed to inactivated virus. CMV p52 antigen expression (575 cells) was observed beginning at 72 hours compared to a background staining of 77 cells that were exposed to inactivated CMV. Three hundred seventy nine and 150 p52 positive cells were present at 96 and 168 hours, respectively. The UV-irradiated CMV exposed cells had counts of 34 and 76 cells (96 and 168 hours, respectively). Positive counts in the BJAB-B1 cells exposed to inactivated CMV represents non-specific staining. 10,000 cells were collected and analyzed per experimental group. The flow cytometry data is summarized in Table 3. The amount of CMV positive BJAB-B1 cells, as also observed in the real-time data, show a decrease with respect to time. Both the transcript data as well as the p52 antigen expression data show that the BJAB-B1 cells are susceptible to a CMV superinfection and there was not a high amount of infection. Qualitative analyses of the immunofluorescent staining in BJAB-B1 and P3HR-1 cells also show that a low amount of infection was occurring.

CMV undergoes an abortive infection in BJAB-B1 cells

A CMV abortive infection was not determined for the P3HR-1 cells. In order to determine if CMV was undergoing an abortive or replicative infection, separate CMV superinfection experiments were performed. Cells were washed after 5 hours incubation with CMV and fresh media was added. The CMV infections were carried out to 10 days. Supernatants were taken immediately after washing to show absence of unattached CMV. Aliquots were taken at 24 and 48 hours for immunofluorescence analysis to confirm CMV infection (data not shown). After 10 days the cells were vortexed and supernatant was collected and placed into cultures of MRC-5 fibroblasts. MRC-5 infections were carried out to 10 days. No CPE was observed in any of the fibroblast infections, suggesting that an abortive infection was occurring in the BJAB-B1 cells.

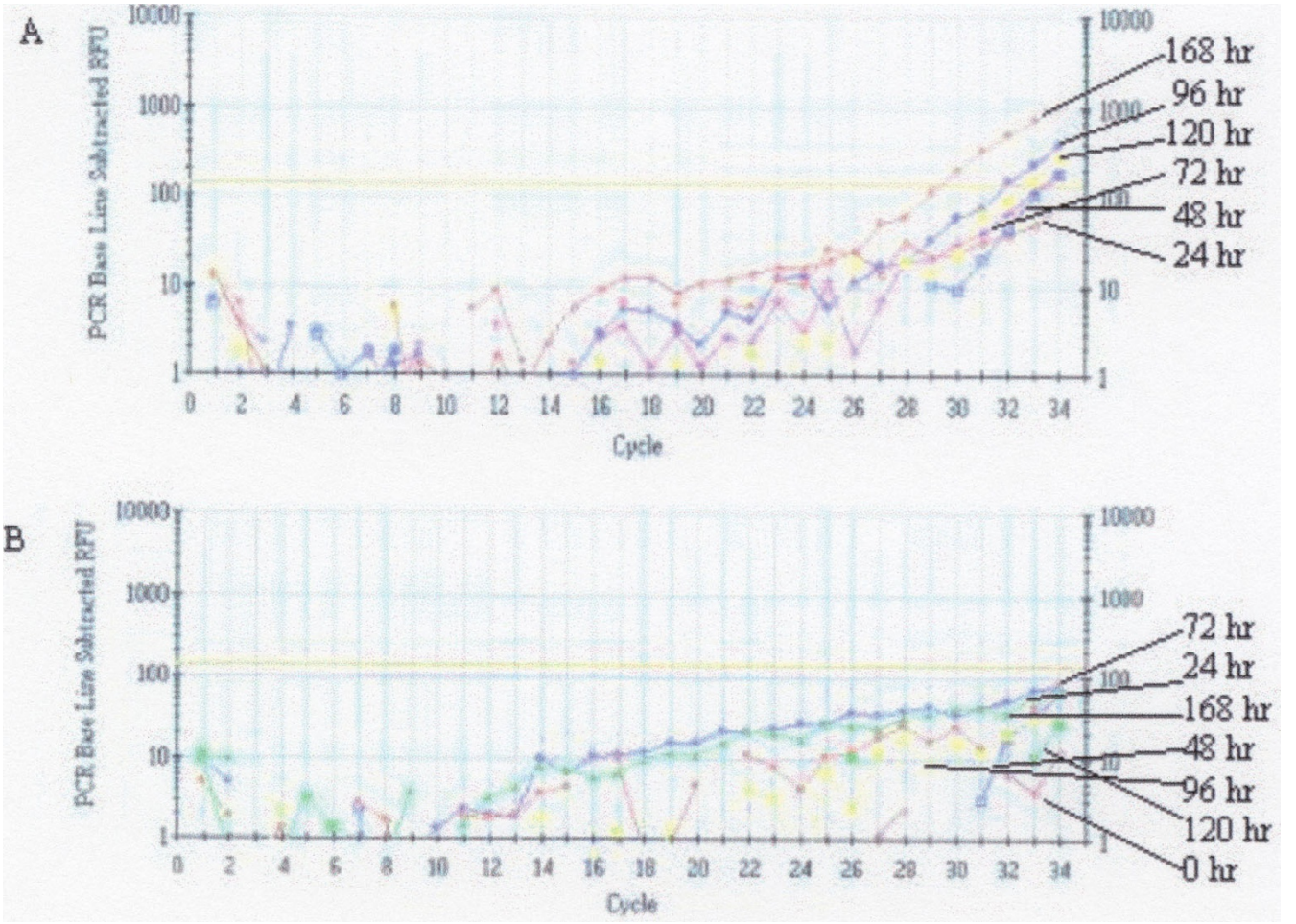

Figure 2
Real-time PCR of the CMV MIE transcript in P3HR-1 cells. In all graphs, the axes are relative fluorescence units (RFUs) versus cycle number. CMV DNA copy standards were run in order to establish a standard curve for quantitative analysis. A1 (500,000 copies), A2 (5,000 copies), A3 (50 copies), and A4 (negative control) (data not shown). cDNA from the P3HR-1 cells were treated similarly to the BJAB-B1 cells. Shown are P3HR-1 cells superinfected with CMV (A) and cells exposed to UV-irradiated CMV (B). Uninfected cells were negative for any CMV transcript (data not shown). Time points are designated in the figure. For these set of experiments, 0, 24, 48, 72, 96, 120, and 168 hours post superinfection were performed. Agarose gel analyses were not performed.

EBV reactivation after CMV superinfections of BJAB-B1 and P3HR-1 cells

This project has shown that the BJAB-B1 and P3HR-1 cells were susceptible to a CMV superinfection by detecting viral transcript and protein. The effect on EBV latency was next examined. The BZLF1 gene product, Zebra (Zta) is an indicator for EBV reactivation. PMA and ionomycin (1 μg/ml) non-specifically reactivates EBV and was used as a positive control for BZLF1 transcript expression in the cell lines. The BJAB-B1 and P3HR-1 cells were incubated with PMA/ionomycin (1 μg/ml) (Figures 5A and 6A, respectively). Presence of the BZLF1 transcript in the superinfected P3HR-1 cells was observed beginning at 24 hours post superinfection, decreased at 48 hours and then was observed to increase after 72 hours (Figure 6B). Detection of BZLF1 in the P3HR-1 cells was performed by real-time PCR only. BZLF1 transcript in the BJAB-B1 cells was first observed at 48 hours post CMV superinfection and was expressed through to 96 hours, as shown in the real-time PCR data (Figure 5B). The amount of BZLF1 transcript expression in the BJAB-B1 cells started to decrease after 48 hours. Agarose gel analysis confirms the real-time PCR data and shows that the PCR was specific (Figure 6D). Due to the relatively low amount of the BZLF1 PCR product in the real-time data and gel analysis, it was expected that the immunofluorescence staining would be minimal. Immunofluorescence analysis was performed only with the BJAB-B1 cells and the experimental group that was su-

Table 1: CMV quantities of standards and superinfected BJAB-B1 cells.

	Type	Threshold Cycle (TC)	Starting Quantity (SQ)
A1	Standard	17.493	5.00×10^5
A2	Standard	23.859	5.00×10^3
A3	Standard	28.712	5.00×10^1
C7	Unknown	32.02	4.07×10^0
C8	Unknown	36.175	1.34×10^{-1}
C9	Unknown	35.825	1.79×10^{-1}
C10	Unknown	35.21	2.96×10^{-1}
C11	Unknown	35.139	3.14×10^{-1}

Data presented here were calculated from the standard curve with the included analysis software. C7, C8, C9, C10, and C11 corresponded to the time points 24, 48, 72, 96, and 168 hours. The TC (threshold cycle) is the cycle number in which the fluorescence of the sample crosses the threshold line. The threshold level is a subjective analysis by placing the line when the samples were in its exponential phase of the PCR reactions. The software calculates the 'Starting Quantity' (SQ), which represents the input copies of cDNA.

Table 2: CMV quantities of superinfected P3HR-1 cells.

	Type	Threshold Cycle (TC)	Starting Quantity (SQ)
A1	standard	17.63	5.00×10^5
A2	Standard	24.14	5.00×10^3
A3	Standard	29	5.00×10^1
D5	Unknown	31.75	1.98×10^0
D6	Unknown	32.59	9.01×10^{-1}
D7	Unknown	29.19	2.17×10^1

Data presented here were calculated from the standard curve with the included analysis software. D5, D6, and D7 correspond to 96, 120, and 168 hours. The T_C (threshold cycle) is the cycle number in which the fluorescence of the sample crosses the threshold line. The threshold level is a subjective analysis by placing the line when the samples were in its exponential phase of the PCR reactions. The software calculates the 'Starting Quantity' (SQ), which represents the input copies of cDNA.

perinfected with CMV was observed to have expression of the BZLF1 protein at 168 hours (Figure 7A). BJAB-B1 cells stimulated with PMA/ionomycin were used as a positive IF control (Figure 7B). Uninfected and UV-irradiated CMV treated cells showed no expression of the BZLF1 transcript or protein indicating no EBV reactivation (data not shown). Mock RT-PCRs were not performed for the BZLF1 PCR reactions because the primers recognize mRNA.

Table 3: Flow cytometry data of p52 antigen within BJAB-B1 cells.

	72 hr	96 hr	168 hr
CMV	575	379	150
uvCMV	77	34	76

Flow cytometry was performed on uninfected cells, cells infected with CMV, and cells incubated with UV-irradiated CMV. The background fluorescence of the uninfected cells was subtracted from the respective samples to give a final quantity of CMV positive cells. 'CMV' indicates BJAB-B1 cells infected with viable CMV and 'uvCMV' indicates cells incubated with UV-irradiated virus. Time points are as indicated. Quantitative values are per 10,000 cells.

Glyceraldehyde-3-phosphate dehydrogenase (G3PDH)

Amplification of the housekeeping gene G3PDH served as a control for RNA integrity. The product was analyzed by agarose (2%) gel electrophoresis and stained with ethidium bromide. This transcript was present at all time points among all experimental groups for both cell lines (data not shown).

Discussion

This study has shown that the BJAB-B1 and P3HR-1 cell lines were susceptible to a CMV superinfection, and a low amount of infection was observed. Numerous studies have been able to show that PBMCs and B-cells in particular can have expression of viral transcript and/or antigen [35–41] however, other studies have been contradictory [40,42] and therefore no consensus among the observations noted. Early studies have shown that in other B-cell lines, CMV DNA has been observed [43–45]. The MOI used in this study (20) indicates that there were 20 virus particles per BJAB-B1 or P3HR-1 cell. This high MOI would have seemed to ensure that enough CMV was present to superinfect the respective cell lines. However, despite the high MOI, the real-time PCR and immunofluorescent staining data from the BJAB-B1 and P3HR-1 cells show low amounts of infection present.

The concept of this study was to show evidence that CMV can superinfect these B-cell lines and may induce EBV reactivation by using a standardized CMV preparation. Infectivity differences have been observed between clinical isolates of CMV and laboratory adapted strains. The strain used in this study, AD169, and the Towne strains were both used to develop vaccines [49]. Differences in infectivity have been observed between 2 of the same strains of CMV and this may be attributed to the deletion or mutation of certain tropism genes upon extensive serial propagation [50,51]. Clinical isolates (wild-type CMV) can carry more than 220 genes, and some of these genes can

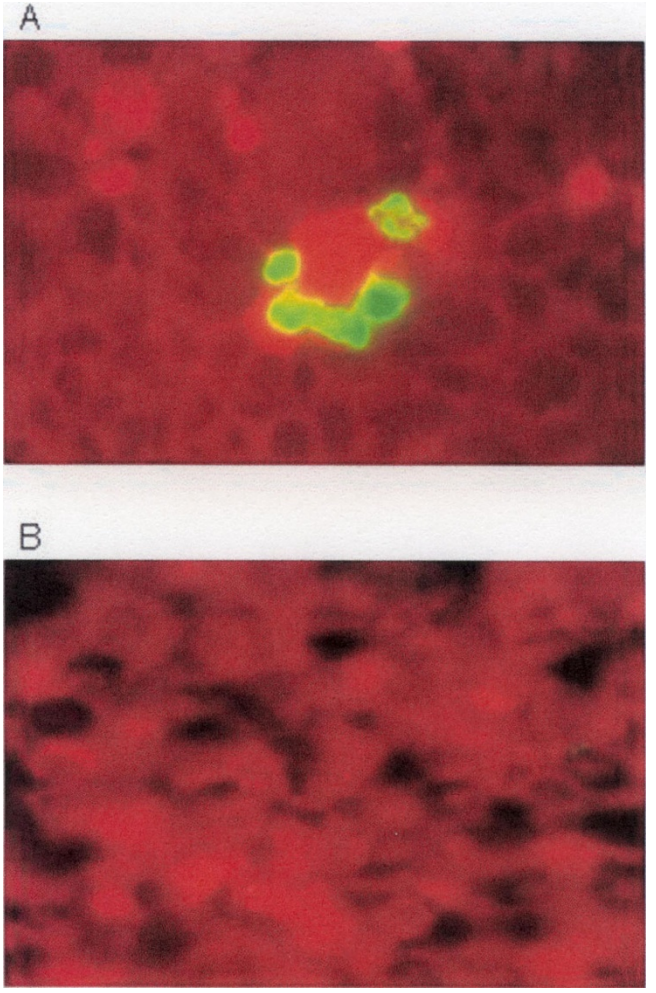

Figure 3
Immunofluorescence of p52 antigen in BJAB-B1 cells superinfected with CMV. Immunofluorescence staining was performed to give a qualitative analysis of CMV protein expression and to further show susceptibility of infection. Approximately 50,000 BJAB-B1 cells for each experimental group were subjected to a cytospin for antigen staining. The cells were fixed in acetone and methanol (1:1) mixture and stained with the appropriate dilution of the FITC labelled antibody. Presence of the p52 antigen was positive at all time points (24, 48, 72, 96, and 168 hours), slide shown is at 24 hours post CMV superinfection. Uninfected cells and cells incubated with UV-irradiated CMV showed no presence of the p52 antigen (data not shown). Positive control slides (Chemicon, Temecula, CA) were stained with the antibody to show that the antibody was working (data not shown).

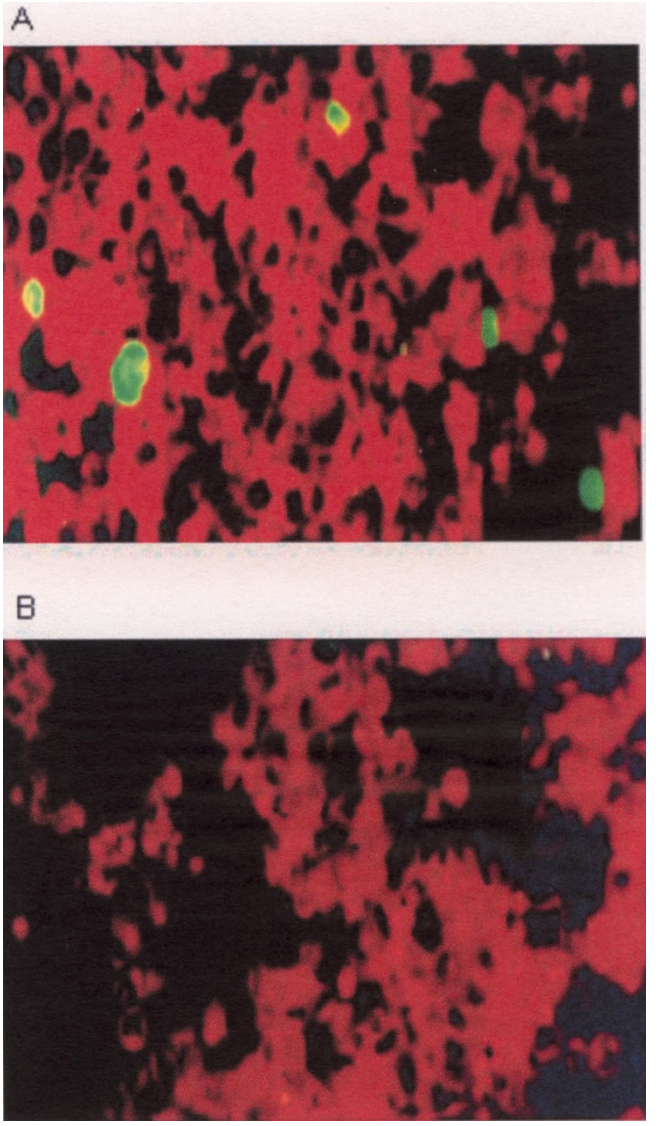

Figure 4
Immunofluorescence of p52 antigen in P3HR-1 cells superinfected with CMV. Immunofluorescence staining was performed to give a qualitative analysis of CMV protein expression and to further show susceptibility of infection. Approximately 50,000 P3HR-1 cells for each experimental group were subjected to a cytospin for antigen staining. The cells were fixed in acetone and methanol (1:1) mixture and stained with the appropriate dilution of the FITC labelled antibody. Presence of the p52 antigen was positive at all time points (24, 48, 72, 96, 120, and 168 hours), slide shown is at 24 hours post CMV superinfection. Uninfected cells and cells incubated with UV-irradiated CMV showed no presence of the p52 antigen (data not shown). Positive control slides were stained with the antibody to show that the antibody was working (data not shown).

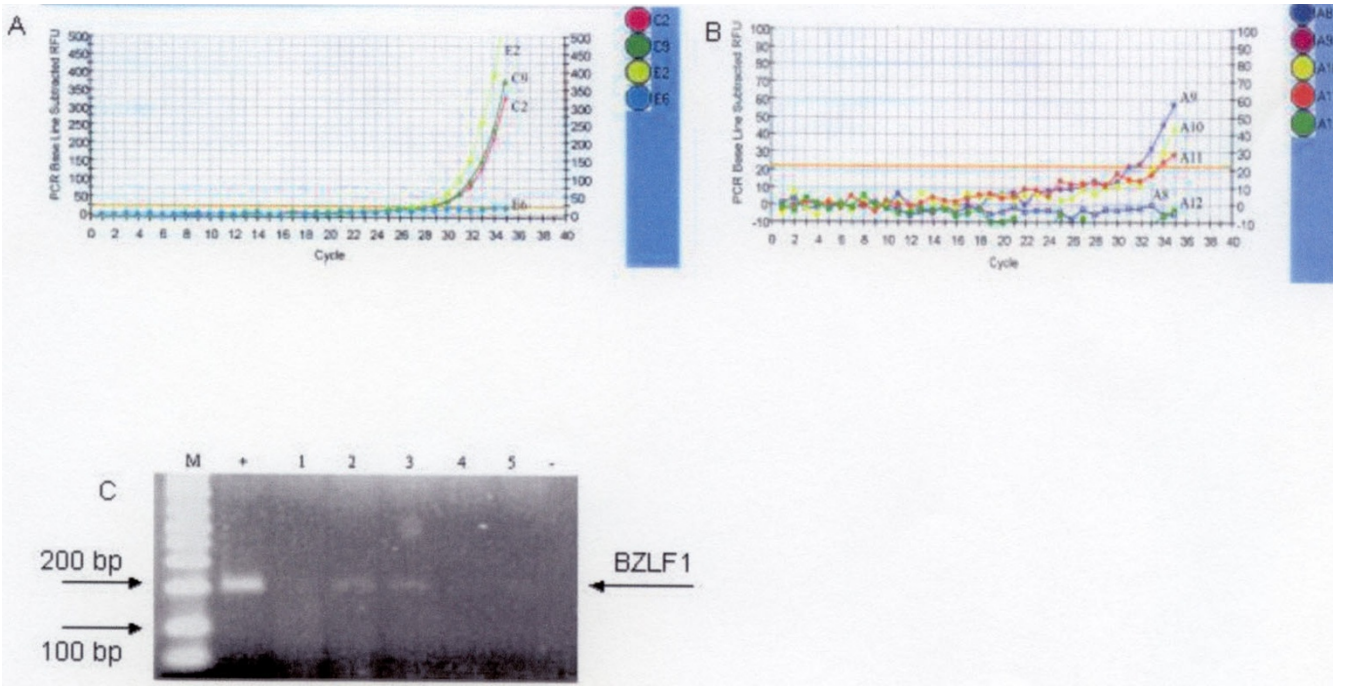

Figure 5
BZLF1 Transcript Expression in BJAB-B1 Cells. BZLF1 transcript was chemically induced with PMA/ionomycin (1 μg/ml) as a positive control for EBV reactivation. Qualitative real-time PCR was performed to show positive expression of the BZLF1 transcript. Time points were taken at 24 hours (C2), 48 hours (C9), and 72 hours (E2). E6 represents unstimulated cells and also served as a negative control (A). Real-time PCR amplification of the BZLF1 transcript was performed using 5 μl of cDNA from the RT reactions among BJAB-B1 cells superinfected with CMV. Time points are at the given designations A8 (24 hours), A9 (48 hours), A10 (72 hours), A11 (96 hours), and A12 (168 hours) (B). Agarose (2%) gel electrophoresis of the real-time PCR samples was performed in order to show specificity of the PCR reactions (C).

be lost by deletion and/or rearrangement during laboratory passage [50]. The AD169 strain used in this study has been passaged many times and because of these variations observed among the CMV strains, this may partly account for the low infectivity observed in the BJAB-B1 and P3HR-1 cells. Perhaps an increase in CMV infection in the cell lines could have been obtained with the use of a clinical isolate of CMV. Our laboratory is currently collecting clinical isolates of CMV for future superinfection experiments.

The first step in CMV infection is attachment to its cellular target; however, the receptors for CMV have not yet been delineated. Heparin sulfate and CD13 have been suggested to be involved in CMV attachment [52–54]. Heparin sulfate is a receptor present on many cell types, but CD13 is not expressed on B-cells. Even with these receptors being implicated as requirements for CMV adherence and penetration, the wide range of cellular tropism observed would suggest that additional receptors might be used.

The detection of CMV protein was performed to further show CMV infection in the cell lines used. The CMV p52 antigen is expressed in the early phase of infection and in the nuclei of infected cells. During a replicative infection, expression of this protein continues through to the late phase of infection [56]. One function of the p52 protein is to aid in replication by binding to the viral DNA polymerase. The immunofluorescence data confirms the real-time PCR data showing that BJAB-B1 and P3HR-1 cells were susceptible to a CMV superinfection and a low amount of infection was observed. The p52 expression as shown in the flow cytometry data of the BJAB-B1 cells also indicate that a low amount of infection was observed. The numbers of p52 positive cells was also observed to decrease with respect to time, perhaps indicating an abortive infection may be occurring. No CPE was observed in any of the fibroblast superinfection studies indicating a lack of extracellular CMV infectious particles. We did not test for cell associated CMV in these experiments, so it is possible that virus emerging from the infected B-cell line may be bound to other cells in the culture. However, the fact that only a small percentage of the B-cells showed evidence of CMV infection along with the lack of virus in the supernatant supports the probability that the CMV infection of the BJAB-B1 cells represents an abortive infection. The data represented here support the work of others showing

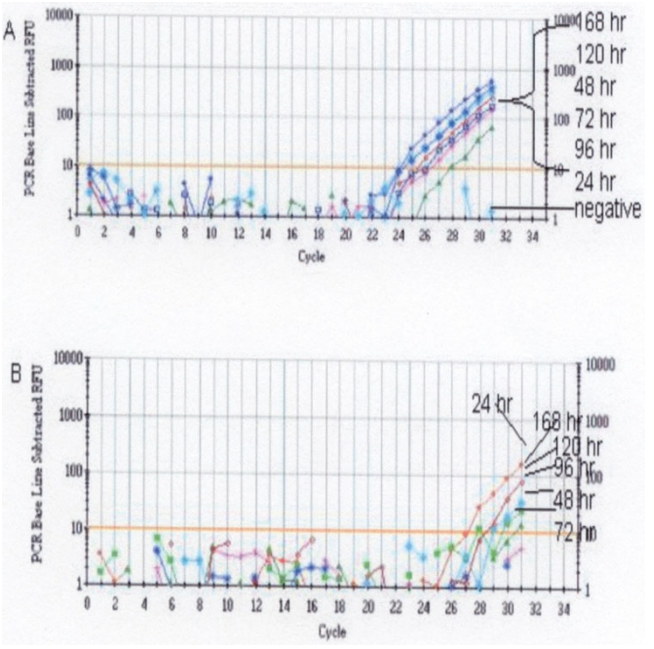

Figure 6
BZLF1 transcript expression in P3HR-1 cells. BZLF1 transcript was chemically induced with PMA/ionomycin (1 μg/ml) as a positive control for EBV reactivation. Qualitative real-time PCR was performed to show positive expression of the BZLF1 transcript. Unstimulated cells served as a negative control (A). Real-time PCR amplification of the BZLF1 transcript was performed using 5 μl of cDNA from the RT reactions among P3HR-1 cells superinfected with CMV (B). Time points are indicated in both (A) and (B).

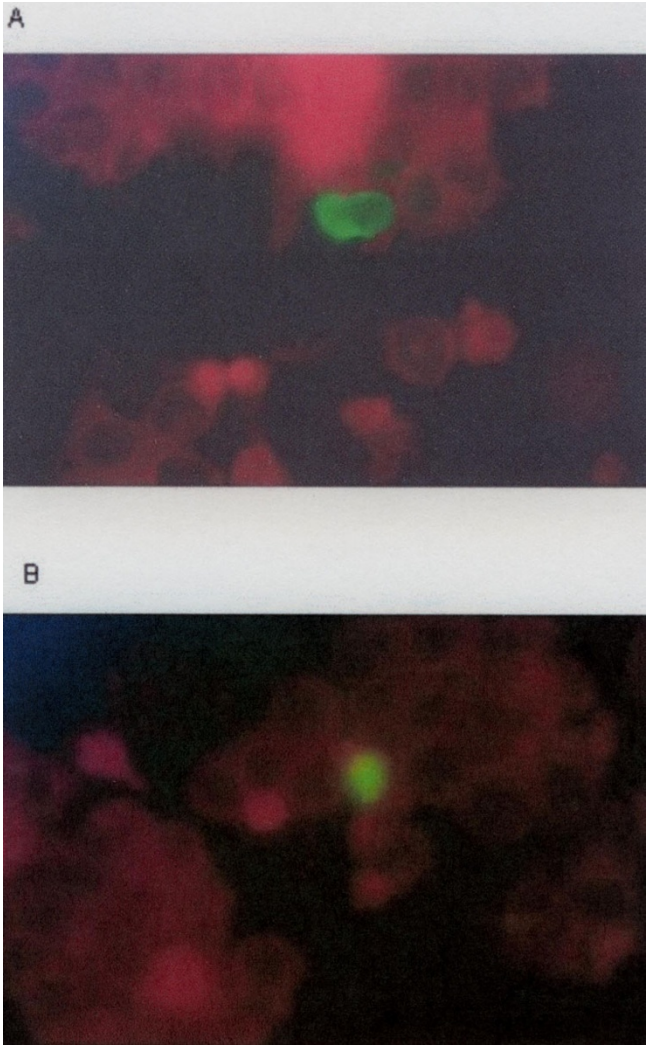

Figure 7
Immunofluorescence of the BZLF1 protein in BJAB-B1 cells. Immunofluorescence staining was performed to show production of viral protein, indicative of EBV reactivation. Stimulated cells and approximately 50,000 experimental sample cells were subjected to a cytospin and prepared similarly to the p52 staining protocol. A secondary FITC-labelled goat-anti-mouse antibody was used because the primary antibody was unlabelled. BJAB-B1 cells were stimulated with PMA/ionomycin (1 μg/ml) for the induction of the BZLF1 protein and for a positive control (A). Presence of the BZLF1 protein was observed within the CMV superinfected BJAB-B1 cells and only at 168 hours (B)

that CMV may undergo an abortive infection in B-cells rather than a productive one [35,36,46–48].

Regardless of whether viable CMV or UV-irradiated CMV is used, the virus still can attach to the host cell and cause the upregulation of cellular genes, including proto-oncogenes [55,57–59]. It was observed in this study that the UV-irradiated CMV had no effect on BZLF1 transcript expression, suggesting that the effect observed in the superinfected BJAB-B1 and P3HR-1 cells was not attachment mediated and infectious CMV is at least required. At this point it has not been determined if CMV acts directly or indirectly to cause EBV reactivation. A direct effect would imply that a CMV transactivator is produced that could somehow cause the expression of or at least aid in the activation of the BZLF1 gene. Flamand et al. observed that HHV-6 (same subfamily as CMV) could cause the reactivation of EBV [33]. The authors suggested that HHV-6 was having a direct effect on EBV reactivation because they were able to observe the presence of both viral antigens in the same cell. However, this observation does not necessarily suggest direct activation because the effect cellular activation may have on EBV latency is not known. There may be some as yet unknown host cell gene or genes that are activated or upregulated in association with HHV-6 penetration and infection. Obviously the same uncertainties can be applied to the CMV superinfections of the BJAB-B1 and P3HR-1 cells.

The BZLF1 gene of EBV is an immediate early gene involved in the first step of EBV reactivation, and its activity is controlled at the transcriptional level [60–66], by protein-protein interactions [64–68], and phosphorylation [64,69,70]. Perhaps CMV may cause the activation of certain cellular kinases, which in turn may activate BZLF1 transcript expression. CMV has been shown to cause calcium flow and protein kinase C (PKC) activation that could then be involved in the early activation of transcription factors [71]. Cellular levels of secondary messengers inositol 1,4,5-triphosphate and 1,2-diacylglycerol [71–73], as well as the MAP kinases (ERK1 and ERK2) [74,75] are also known to be upregulated, which may have an effect on EBV reactivation. One study performed a DNA microarray analysis on CMV infected cells and observed an upregulation of 258 cellular mRNAs whose level changes were by a factor of 4 or more after the onset of viral replication [76]. The functions of some of these genes still remains highly speculative, yet it gives an insight as to what potential roles these genes may play, particularly in EBV reactivation.

The CMV transcript expression in BJAB-B1 cells was observed beginning at 24 hours and was expressed through to 168 hours. The BZLF1 transcript was observed beginning at 48 hours and expressed though 96 hours. There was a greater amount of CMV MIE transcript present compared to the BZLF1 transcript in the BJAB-B1 cells. BZLF1 protein expression was observed only at 168 hours post CMV superinfection. CMV p52 protein expression was observed through 168 hours post superinfection (immunofluorescence and flow cytometry data) and p52 positive cells peaked at 72 hours (flow cytometry). Among the P3HR-1 cells, the CMV transcript was also expressed from 96 hours to 168 hours; however, the viral load increases with respect to time as opposed to the BJAB-B1 cells. During the earlier time points (24, 48, and 72 hours), the sample curves have not yet crossed the threshold and therefore were not included in the program's final quantitative analysis. The BZLF1 transcript in the superinfected P3HR-1 cells shows presence at 24 hours, decreases at subsequent time points and finally peaks at 168 hours. Differences in the CMV and BZLF1 transcript expression between the BJAB-B1 and P3HR-1 cells may be attributed to the fact that these are different cell lines, despite the presence of EBV. The kinetic expression of both viral transcripts and proteins in the respective cell lines would seem to suggest that CMV may be having an indirect effect on EBV reactivation through the participation of cellular factors.

Despite the low infectivity of the BJAB-B1 and P3HR-1 cells by CMV, EBV reactivation was observed in the experimental groups superinfected with CMV. The cell lines that were exposed to UV-irradiated CMV had no indication of BZLF1 transcript or protein suggesting that the effect on EBV reactivation was not attachment mediated and that infectious CMV was required. *In vivo*, it would be difficult to pinpoint one specific factor to cause the switch from latency to lytic infection. However, *in vitro*, it was most interesting to observe EBV reactivation only in the cell lines that were superinfected with CMV.

A CMV infection, *in vivo*, can contribute to the net immunosuppressive status of the immunocompromised patient [11]. A CMV infection can result in the production of certain immune effector proteins (cytokines and chemokines) that may affect latency in the EBV infected B-cells [77]. The production of these cytokines and chemokines from a CMV superinfected BJAB-B1 or P3HR-1 cell may affect neighboring cells and therefore cause EBV reactivation, implying that CMV could be having an indirect effect on EBV latency.

Conclusions

Because CMV infection and disease is such an important concern among the immunosuppressed patient populations (transplant and HIV infected individuals), its involvement in the reactivation of EBV must be considered. This report has shown that BJAB-B1 and P3HR-1 cells, both latently infected with EBV, were susceptible to a CMV superinfection and only in the experimental groups that were superinfected with CMV were there any indication of EBV reactivation. These data support the clinical studies showing an *in vivo* relationship between a CMV active infection and EBV reactivation. Although it would be very difficult to prove that a CMV infection is the cause of EBV reactivation in clinical situations due to the gamut of factors that are involved, the fact that EBV reactivation was observed after *in vitro* CMV superinfections supports this role. Inducers of EBV replication are of importance because of the serious complications that are associated with recurrent EBV infections, particularly in immunosuppressed individuals. Since a majority of the population is seropositive for CMV, reactivation of CMV in immunosuppressed patients may further accentuate the reactivation of EBV therefore contributing to the proliferation of EBV infected B-cells. The results in this project suggest that CMV may contribute as a co-factor in EBV pathogenesis. More detailed molecular studies are needed to examine the direct and/or indirect effects CMV may have on EBV.

Materials and Methods

Cells and virus

P3HR-1 (ATCC) and BJAB-B1 cells (generously donated by Dr. George Miller) are EBV latently infected B-cells kept in RPMI 1640 medium (supplemented with 10% FBS and an antibiotic-mycotic solution) (Gibco, Invitrogen Corp., Carlsbad, CA). The AD169 laboratory strain of CMV was from the clinical virology laboratory (Tampa General

Hospital, Tampa, FL), and used for the superinfections. CMV was grown and harvested from MRC-5 fibroblast cultures (Biowhittaker, Walkersville, MD) and concentrated by ultra-centrifugation (28000 g for 60 minutes at 4°C). The CMV titer was determined using the shell vial assay. Briefly, this is an immunofluorescence assay in which 1:10 dilutions of the virus were prepared in shell vials and incubated at 37°C overnight. The MRC-5 cells in the shell vials were fixed and permeabilized for 15 minutes in methanol:acetone (1:1) and then washed in PBS (Bartels Inc., Intracel Corp., Frederick, MD, reconstituted in 1 liter of Millipore water, pH 7.0–7.6). The cells were stained with a FITC labelled antibody directed against the major immediate early antigen (MIE) (Chemicon, Temecula, CA) and visualized using a fluorescent microscope. Each green (positive) cell was counted as one viral particle.

CMV superinfection of the BJAB-B1 and P3HR-1 cells

500,000 cells were counted using the Cell-DYN 4000 (Abbott Systems, Abbott Park, IL) and placed in a 24-well culture plate with RPMI 1640 medium. The P3HR-1 and BJAB-B1 cells were infected at a multiplicity of infection (MOI) of 20 and were either uninfected, infected, or exposed to UV-irradiated virus. CMV was exposed to UVC light for 2 hours in our biological safety cabinet. The experiments were carried out for 7 days and samples were taken at 24, 48, 72, 96, and 168 hours. Time points for the P3HR-1 cells were the same except for an additional time point at 120 hours. At each time point, cells were analyzed by immunofluorescence and real-time reverse transcriptase-polymerase chain reaction (RT-PCR). The P3HR-1 cells were not subjected to flow cytometric analysis. Superinfections were repeated three times and data shown are representative experiments.

MRC-5 fibroblast infection of supernatants from CMV superinfections

Separate superinfection experiments of the BJAB-B1 cells were set up and were incubated with CMV for 5 hours to allow for viral attachment. The cells were then washed to remove any unattached virus and fresh medium was added. The CMV superinfections were carried out to 10 days. The cultures were vortexed, supernatant was collected, and added to cultures of MRC-5 fibroblasts. Supernatant fluid was collected immediately after infection and washing to establish that any unattached CMV was not present. MRC-5 fibroblasts were examined for CPE.

Immunofluorescent staining for p52 (CMV) and BZLF1 (EBV)

Aliquots of 100 µl (approximately 50,000 cells) from each experimental group were subjected to a cytospin and fixed and permeabilized similarly to the shell vial assay. The anti-p52 and anti-BZLF1 antibodies (Dako Corporation, Carpinteria, CA) were prepared according to package inserts. Evan's Blue was added to the final p52 solution. The p52 antibody was directly labelled with FITC (fluorescein isothiocyanate) and did not require a secondary antibody step. The BZLF1 antibody is unlabelled and required a secondary goat anti-mouse antibody labelled with FITC. The secondary antibody was diluted 1:200 in PBS and Evan's Blue was added. The primary and secondary antibodies were incubated in a humidified chamber at 37°C for 30 minutes. The slides were washed in PBS after each incubation step, prepared with mounting media, and visualized under a fluorescent microscope.

Flow cytometry of p52 antigen

Aliquots of 300 µl (approximately 150,000 cells) were placed in 5-ml Falcon tubes and washed in PBS. The cells were fixed and permeabilized with pre-made solutions from Caltag Laboratories (Burlingame, CA) (Fix & Perm) according to kit instructions. During the permeabilization step, FITC-labelled antibody (p52) was added at a 1:5 dilution. Cells were washed in PBS and then 750 µl of paraformaldehyde was added and read on the FACScalibur flow cytometer (Becton Dickinson, Los Angeles, CA). Fluorescence parameters were set with regards to infected and uninfected samples. CMV infected and uninfected MRC-5 fibroblasts were used as positive controls for viable CMV and antibody staining. 10,000 BJAB-B1 cells were first separated according to size and granularity (forward vs. side scatter) on a dot plot. Viable cells were then selected and gated for further analysis on a histogram for FL1 (FITC, p52 antigen) versus cell counts. The final histogram analyses involved subtracting background of the corresponding mock-infected cells from the BJAB-B1 cells superinfected with CMV or UV-irradiated CMV. Analysis was performed using the CellQuest software (Becton Dickinson, Los Angeles, CA). The P3HR-1 cells were not analysed by flow cytometry.

RNA extraction and isolation

The nucleic acid isolation was performed on an automated extractor from the Organon Tekinika Corporation (Boxtel, Netherlands) and the extractions were performed according to kit instructions modified from the method by Boom et al. [78]. All reagents used for extractions were from the company. Aliquots of 400 µl (approximately 200,000 cells) were used per experimental time point. The final result was approximately 50 µl of nucleic acid in elution buffer. The nucleic acid samples were then subjected to DNase treatment and RNA clean up with Ambion's (Austin, TX) DNA-free kit following their provided protocol.

RNA quantitation

Using Ribogreen dye and RNA quantitation standards from Molecular Probes (Eugene, OR), the amount of RNA

in the experimental samples were calculated. Briefly, an aliquot of the RNA samples were diluted 1:100 in TE buffer. RNA standards were prepared (4000, 2000, 1000, 500, 250, and 125 ng). The Ribogreen dye was diluted 1:200 in TE buffer and 20 µl of the diluted RNA (standard or sample) was mixed with 20 µl of the diluted Ribogreen dye. Samples and standards were placed in a microtiter plate and read on the CytoFluor reader from Applied Biosystems (Foster City, CA) (excitation 485 nm/20 nm and emission 530 nm/25 nm). The standard curve and sample quantitations were determined using an Excel spreadsheet.

Reverse transcription

RNA for cDNA production was performed using Promega's (Madison, WI) random hexamers, RNAsin, RT buffer, and RT enzyme. For each sample, the RNA (2 µg) was initially incubated with the random hexamers (0.5 µg) and enough PCR grade water to bring the volume up to 20 µl. Incubation of the RNA and random hexamers was performed at 95°C for 5 minutes and then held at 4°C. The RT mix included 5.6 µl of 5X RT buffer, 0.02 mM of dNTPs (with dTTP, not dUTP, Perkin Elmer, Foster City, CA), 14.22 units of AMV RT enzyme, and 20 units of RNAsin per reaction. A total of 9.68 µl of the RT mix was added to the RNA samples (with the random hexamers) and then subjected to RT cycle conditions (42°C for 45 minutes, 95°C for 5 minutes and a 4°C hold). All incubations were done on BioRad's iCycler (Richmond, CA). Mock RTs were set up in which PCR grade water was substituted for the RT enzyme as a check for DNA contamination.

Real-time PCR

The PCR mix per reaction included 10 µl SYBR Green buffer, 1.5 mM $MgCl_2$, 1 µM dNTP mix (with dUTP), 0.5 µl CMV primer mix, 2.5 units Ampli*Taq* gold, 0.05 units of uracil-N-glycosylase (UNG, Epicentre, Madison, WI) that had been diluted 1:10 in UNG buffer, and molecular grade water to bring the volume up to 45 µl with the addition of 5 µl of cDNA or standard. The PCR reagents were obtained from Perkin Elmer. The CMV primers, from Synthetic Genetics, Inc. (San Diego, CA), amplify the MIE gene. The BZLF1 primers recognize only mRNA and the sequences are 5'-TTCCACAGCCTGCACCAGTG-3' and 5'-GGCAGCAGCCACCTCACGGT-3' [79]. Primers were generated by Sigma Genosys (The Woodlands, TX). The CMV PCR was quantitative and the BZLF1 PCR was qualitative. The PCR consisted of 2 pre-incubation steps, 37°C/15 minutes and 95°C/10 minutes (for UNG activation and UNG denaturation/*Taq* activation). The cycles for PCR were 95°C/30 seconds, 55°C/30 seconds, and 72°C/30 seconds for 35 cycles. CMV DNA copy standards (Synthetic Genetics, Inc.) were used to set up a standard curve. Standards were 500000, 5000, and 50 CMV DNA copies. Dilutions were set up using PCR grade water. The software (Bio-Rad, Richmond, CA) calculated the standard curve as well as determined the amount of CMV DNA in the superinfected samples. Agarose (2%) gel electrophoresis was performed in order to determine the specificity of the PCR reactions in the BJAB-B1 cells only. Primers and positive control for the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene were from Clontech (BD Biosciences Clontech, Palo Alto, CA). PCR recipe and conditions were run according to the included protocol. G3PDH transcripts from both cell lines were analyzed on a 2% agarose gel only.

Real-time PCR analysis

The instrument camera took numerous fluorescence readings during the annealing stages and displayed the average reading for that particular cycle. At the end of the PCR cycling, the base line fluorescence was calculated and all the samples were normalized to zero. The threshold level (C_T) was set at a point when the standards or positive control were in its respective exponential phase of amplification. The C_T value is the cycle number in which a sample's fluorescence crosses the set threshold level, and therefore is called positive. During quantitative analysis, the standard curve was calculated and the sample concentrations were derived from the curve. All calculations were performed with the included software.

Authors' contributions

RCA performed all assays, drafted the manuscript, and participated in the conception of this project. RHW also participated in the conception as well as real-time PCR and flow cytometry analyses.

All authors have read and approved the final manuscript.

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