Non-replicating Epstein-Barr Virus-based Plasmids Extend Gene Expression and Can Improve Gene Therapy in Vivo*

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To date, no gene transfer vector has produced prolonged gene expression following a single intravenous injection and then efficiently re-expressed the delivered gene following repeated systemic injection into immunocompetent hosts. To overcome these limitations, a gene therapy regimen using non-replicating Epstein-Barr virus (EBV)-based expression plasmids was developed. One plasmid contains the FR (EBV family of repeats) sequence and the expressed gene. The other encodes Epstein-Barr nuclear antigen 1 (EBNA-1), but lacks FR. Although unable to replicate in mice, intravenous co-injection of EBV-based plasmids in cationic liposome-DNA complexes (CLDCs) substantially prolonged luciferase gene expression. The use of a two-vector system limited host exposure to the EBNA-1 gene product. Furthermore, this EBV-based vector system could be intravenously re-injected multiple times into immunocompetent mice without loss of transfection efficiency. Use of this vector system significantly improved the therapeutic efficacy of the biologically important human granulocyte colony-stimulating factor gene. Delivery of the human granulocyte colony-stimulating factor gene in EBV-based plasmids increased circulating white blood counts for at least 2 months following a single CLDC-based intravenous co-injection. Conversely, white blood counts were never elevated following injection of CLDCs lacking EBV-derived elements. Thus, this EBV-based plasmid vector system both markedly prolongs gene expression at therapeutic levels and efficiently and repeatedly re-transfects immunocompetent hosts. These properties of EBV-based plasmid vectors appear to be due, at least in part, to the documented abilities of the EBNA-1 protein both to retain FR-containing DNA intracellularly and within the nucleus and to block anti-EBNA-1 cytotoxic T cell responses.

Although some recombinant viral vectors can produce long-term gene expression following a single in vivo administration (1, 2), anti-vector immune responses have severely limited their ability to re-express genes in immunocompetent hosts (3–6). Plasmid-based vectors generally produce only short-term gene expression, apparently due largely to the rapid elimination of intracellularly delivered plasmid DNA (7, 8). Replicating episomal vectors, which incorporate a viral DNA origin of replication and a viral early gene product that binds to the origin, can extend reporter gene expression (9, 10). Human papovavirus-based and modified SV40-based vector systems have been shown to prolong significantly the duration of luciferase gene expression as well as to replicate in mice following CLDC1-based gene delivery in vivo (9, 10). More recently, a single Epstein-Barr virus (EBV)-based plasmid vector containing both the latent origin of replication (oriP) of EBV and EBNA-1 has been shown to prolong the duration of luciferase and β-galactosidase gene expression following a single intrahepatic injection in mice (11). However, to do so, each of these vectors requires prolonged expression of either large T antigen or EBNA-1, which can potentially produce oncogenic (10, 12) and, in the case of large T antigen, toxic immune responses (13, 14) following its expression in vivo. Furthermore, replication-based episomal vectors appear to depend on the presence of actively dividing cells (10), which comprise only a small fraction of cells present in post-natal hosts. We have shown that an EBV-based two-plasmid system can both significantly prolong the duration of gene expression produced by plasmid vectors and facilitate efficient repeated transfection in fully immunocompetent mice. One plasmid bears the FR (EBV family of repeats) sequence within the expression cassette, but lacks the region of dyad symmetry (referred to as DS) of oriP of EBV. The other contains the EBNA-1 gene, but lacks FR. EBNA-1 is a DNA-binding protein that binds in a site-specific fashion to sequences within EBV oriP. EBV oriP consists of two non-continuous regions: FR, which contains (1–20) tandem imperfect copies of a 30-base pair sequence, and DS, which contains a 65-base pair element and is separated by ~1000 base pairs from FR (15). The presence of both DS and FR are required in cis for stable replication (15–18). In the

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1 The abbreviations used are: CLDC, cationic liposome-DNA complex; EBV, Epstein-Barr virus; EBNA-1, Epstein-Barr nuclear antigen 1; CMV, cytomegalovirus; HCMV, human cytomegalovirus; hG-CSF, human granulocyte colony-stimulating factor; DOTIM, 1-[2-(9Z-octadecenoyloxy)ethyl]-2-[8Z-heptadecenoyl]3-[2-hydroxyethyl]-imidazolinium chloride; MLVs, multilamellar vesicles; DOTMA, N1,1-dioleoylpropyl-N,N,N,N-trimethylammonium chloride; ITR, inverted terminal repeat; CAT, chloramphenicol acetyltransferase; ANCs, absolute neutrophil counts.
presence of EBNA-1, plasmids containing FR but lacking DS demonstrate enhanced retention both within cells and in the nucleus (19–21), but are unable to replicate stably (15–18). FR lies within a matrix attachment region identified on the EBV nucleus (19–21), but are unable to replicate stably (15–18). We hypothesized that the ability of FR to mediate plasmid retention intracellularly might extend the duration of expression of genes delivered by CLDCs since CLDCs transfect non-dividing cells with DNA that exists as episomes in vitro (23), and intravenously injected CLDCs transfect primarily vascular endothelial cells (24, 25), which are largely non-dividing in normal adult mice (26, 27).

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Purification**—Plasmid p4395, CMV-EBNA-1, was constructed by isolating the HindIII/AccI DNA fragment of p630 (21) and inserting it by blunt end ligation into the EcoRV/ BamHI site of pSP72 (28), a gift from F. Feig and R. Zaugg (Vical). Plasmid p4329, CMV-luc-FR-1, was constructed by partially digesting p985 (29) with BamHI and then with KpnI and then ligationing the ~3-kilobase pair DNA fragment containing FR sequences upstream of the thymidine kinase promoter linked to the luciferase cDNA into the BamHI/KpnI fragment of p4190 (30). Plasmid p4379, CMV-luc-FR-2, was constructed by digesting p885 with BamHI, isolating the ~0.9-kilobase pair DNA fragment containing FR, and ligationing it into the BamHI site (3′ to the luciferase cDNA) of VPRI255. Plasmid p4458, a single plasmid containing CMV-CMV-EBNA-1-CMV-luc-FR-2, was constructed by digesting p4379 with XmnI and excising the 3.5-kilobase fragment containing the full expression cassette (CMV-intron-EBNA-1-poly(A) fragment) from p4395 with XhoI and BglII, filling in, and subsequently ligationing this fragment by blunt end ligation into the XmnI site of p4379. Plasmid p4402, CMV-G-CSF-FR, was constructed by first inserting the 0.9-kilobase pair BamHI DNA fragment from p985 containing FR into the site of VR1223 (28) from Vical) and then replacing the 1.7-kilobase pair Pertalbal luciferase cDNA from VR1223 with a HindIII/SalI fragment containing the 650-base pair hG-CSF cDNA from p4195 (30) by blunt end ligation. Plasmids were purified using alkaline lysis and ammonium acetate precipitation as described previously (30).

**Preparation of Caticionic Liposomes and CLDCs—DOTIM/Cholesterol** MLVs were prepared as described previously (24). CLDCs were prepared as described (30).

**In Vivo Transfections and Analysis of Gene Expression**—Groups of four female ~25-g ICR, BALB/c, or C57Bl6 mice (Simonson, Gilroy, CA) were injected intravenously with 200 μl of 5% dextrose in water containing 960 μmol of DOTIM/cholesterol MLVs (24) composed to a total of 60 μg of plasmid DNA. Mice injected intravenously with pure DOTMA MLVs received a total of 1040 μmol of DOTMA liposomes complexed to 60 μg of plasmid DNA. Mice were killed at various times post-CLDC injection; samples of lung and heart tissues were analyzed for luciferase activity (24) as described previously, or hG-CSF activity in serum was determined by enzyme-linked immunosorbent assay as described previously (30). Total white blood cell counts were determined with a hemocytometer using EDTA-anticoagulated blood diluted in a 1:10 dilution of phosphate-buffered saline (0.01 mol/liter, pH 7.4). For EBNA-1, microwave antigen retrieval was used with 0.01 mol/liter citrate acid buffer (pH 6.0) for 10 min. Rat monoclonal antibody against EBNA-1, clone 1H4-1 (gift generously provided by Dr. Bill Sugden and incubated at a dilution of 1:40 overnight at 4°C. Mouse anti-p53 monoclonal antibody (Santa Cruz Biotechnology Inc.) was diluted to 1:100 and also incubated overnight at 4°C. Binding was labeled using biotinylated anti-rat (for EBNA-1) and anti-rabbit (for p53) antibodies (Vector Labs, Inc., Burlingame, CA), followed by streptavidin-horseradish peroxidase (Zymed Laboratories Inc., South San Francisco, CA) at 1:200. Peroxidase activity was visualized with diaminobenzidine (Sigma). Sections were counterstained with 1% methyl green.

Double labeling for EBNA-1 and factor VIII was performed using a modified immunoperoxidase assay. In brief, after hydration, antigen retrieval was performed in a citrate buffer with microwaves. Rat anti-EBNA-1 antibody was applied first at a 1:4500 dilution at 4°C overnight. Secondary biotinylated goat anti-rabbit antibody at a dilution of 1:50, an ABC Elite kit, and diaminobenzidine (all provided by Vector Labs, Inc.) were used for signal visualization. Slides were then pre-treated with 0.025% trypsin (Life Technologies, Inc.), rinsed, blocked with normal goat serum, and incubated with anti-factor VIII/von Willebrand factor antibody (Dako Corp., Carpinteria, CA). Slides were incubated with biotinylated goat anti-rabbit antibody, incubated with ABC, and visualized with aminoethylcarbazole (Biomed, Foster City, CA). Slides were rinsed, stained with hematoxylin and eosin, and coverslipped for microscopic analysis. Double labeling for EBNA-1 and cytokeratin was performed as described above, except that after the diaminobenzidine step, Histomouse blockers A and B (Zymed Laboratories Inc.) were applied, followed by a mouse anti-cytokeratin monoclonal antibody (AE1/AE3; Zymed Laboratories Inc.). This was followed by biotinylated anti-mouse antibody (Vector Labs, Inc.), streptavidin-horseradish peroxidase, and AEC chromagen.

**RESULTS**

**Intravenous Co-injection of Luciferase-FR- and EBNA-1-containing Plasmids Significantly Prolongs Luciferase Gene Expression in Mice**—We first assessed whether our EBV-based vector system could prolong gene expression relative to the CMV-luc expression plasmid containing two AAV inverted terminal repeat (ITR) sequences (35). Specifically, we compared the amount of luciferase activity produced both 24 h and 7 days following a single intravenous injection of CLDCs containing (a) CMV-luc-FR-1 plus CMV-EBNA-1; (b) CMV-luc-FR-1 plus CMV-CAV (30); or CMV-luc-AAV-ITR (35) plus CMV-CAT (obtained from Dr. J. Samulski), which contained flanking AAV ITR sequences. The CMV-luc-FR-1 plasmid, whether co-injected with either CMV-EBNA-1 or CMV-CAT, produced similar levels of luciferase activity in lungs and heart 24 h after administration (see Fig. 2A). (The lungs and heart are the organs most efficiently transfected following intravenous injection of CLDCs (9, 24, 25, 30, 36).) At 24 h following injection, CMV-luc-AAV-ITR plus CMV-CAT produced somewhat higher peak levels of luciferase activity in lungs and heart than CMV-luc-FR-1 plus CMV-EBNA-1. However, 1 week after injection, mice co-injected with CMV-luc-FR-1 plus CMV-EBNA-1 expressed significantly more luciferase activity (p < 0.0005) than mice co-injected either with CMV-luc-FR-1 plus CMV-CAV or with CMV-luc-AAV-ITR plus CMV-CAT (see Fig. 2A). Thus, CLDC-based intravenous co-injection of a CMV-EBNA-1 plasmid plus an FR-containing CMV-luc plasmid significantly prolonged luciferase gene expression compared either with the CMV-EBNA-1 plasmid plus a CMV-luc plasmid lacking FR or with a CMV-luc plasmid containing flanking AAV ITR sequences. Although the EBV-based plasmid vector system produced significantly more luciferase activity at day 7 than did...
An Improved Luciferase-FR-containing Plasmid Further Prolongs Expression in Immunocompetent Mice—We noted that p4329, our CMV-luc-FR-1 expression plasmid in which the FR sequences were inserted between the heterologous intron and the luciferase cDNA and which also contained the thymidine kinase promoter downstream of the HCMV promoter, produced peak tissue levels of luciferase activity 5-fold lower than comparable HCMV-based vectors (see Fig. 2A). Since the presence of the thymidine kinase promoter as well as the position of FR within the vector could each potentially interfere with gene expression, we assessed luciferase activity produced by p4379 (CMV-luc-FR-2), an HCMV-IE1-based luciferase expression plasmid containing a 5' heterologous intron in which we inserted FR downstream of the luciferase cDNA in a plasmid containing only the HCMV promoter (Fig. 1). We observed that placement of FR downstream of the coding sequence yielded a vector that produced peak levels of luciferase gene expression comparable to those of the parent HCMV-based vector lacking FR (data not shown).

We then assessed the effects of intravenously co-injecting this more efficient CMV-luc-FR-2 plasmid (p4379) plus p4395, CMV-EBNA-1 (which lacks FR) (Fig. 1), on the duration of luciferase gene expression in mice. A single CLDC-based intravenous co-injection of CMV-luc-FR-2 plus CMV-EBNA-1 produced $1.9 \pm 0.50$ ng of luciferase/mg of tissue protein in the lungs and $0.4 \pm 0.11$ ng of luciferase/mg of tissue protein in the hearts of ICR mice killed 11 weeks later (Fig. 2B). These levels were significantly higher than the luciferase activity produced in the lungs ($0.3 \pm 0.11$ ng of luciferase/mg of tissue protein; $p < 0.005$) and hearts ($0.2 \pm 0.01$ ng of luciferase/mg of tissue protein; $p < 0.05$) of ICR mice killed 8 days after intravenous co-injection of CLDCs containing CMV-luc-FR-2 plus CMV-CAT. We showed that the luciferase activity produced 7 days after CLDC-based intravenous co-injection of CMV-luc-FR-1 plus CMV-CAT was similar to background levels present in un.injected mice (Fig. 2A). Thus, a single CLDC-based intravenous co-injection of a CMV-EBNA-1 plasmid plus an FR-containing luciferase plasmid significantly increased luciferase activity in heart and lungs for at least the next 11 weeks, whereas luciferase activity had returned to very low levels in these organs within 1 week following injection of the FR-containing luciferase plasmid without EBNA-1.

In these experiments, we used a derivative of the HCMV-IE1-driven expression plasmid (28) that produced therapeutic levels of erythropoietin for at least 90 days following a single intramuscular injection of the HCMV-IE1-erythropoietin plasmid alone (37). However, we found that this same HCMV-IE1-based plasmid, in the absence of EBV FR plus the EBNA-1 gene, produced very low levels of luciferase activity even 7 days after a single CLDC-based intravenous injection. Only transient gene expression from similar HCMV-IE1-based expression plasmids following CLDC-based intravenous injection has been reported previously (9, 25, 30, 36).

Repeated Intravenous Re-injection of CMV-EBNA-1 Does Not Limit Expression of Co-injected FR-containing Plasmids—Previously, efficient re-expression of transiently expressed genes following either a single intratracheal (38) or intravenous (30) re-injection of CLDCs into immunocompetent animals has been reported. We assessed whether repeated re-injection of the EBNA-1 gene would limit re-expression of a co-injected reporter gene. To maximize the host’s exposure to the EBNA-1 gene product, we constructed p4458, CMV-luc-FR-CMV-EBNA-1, a single expression plasmid containing both the entire HCMV-EBNA-1 expression cassette from CMV-EBNA-1 and the full-length HCMV-luc-FR expression cassette from CMV-luc-FR-2 (Fig. 1). We used this single combined expression plasmid to determine whether EBNA-1 could mediate prolonged expression of luciferase following repeated systemic injection of the CMV-EBNA-1 plasmid into fully immunocompetent mice.

The first group of mice were pre-injected with CMV-EBNA-1 plus CMV-CAT rather than with CMV-luc-FR-CMV-EBNA-1 to assess the effect of repeatedly injecting CMV-EBNA-1 on the subsequent re-expression of luciferase while avoiding potential anti-luciferase immune responses (39, 40) generated by repeatedly injecting the luciferase gene. Mice that received a total of four prior injections of plasmids expressing EBNA-1, re-injected at 3-week intervals and then a single intravenous injection of CMV-luc-FR-CMV-EBNA-1, showed levels of luciferase activity comparable to those receiving a single injection of CMV-luc-FR-CMV-EBNA-1 3 weeks prior to injection ($p < 0.4$) (Fig. 3). Therefore, the EBNA-1-expressing plasmid was fully capable of mediating the prolonged expression of the co-expressed FR-containing luciferase gene, even after extended and repeated exposure of the EBNA-1 antigen to the immunocompetent host. Thus, the host immune response to repeated exposure to EBNA-1 does not appear to limit the efficacy of this EBV vector system.

We also assessed whether single or repeated intravenous injection of the CMV-luc-FR-CMV-EBNA-1 plasmid into ICR mice would produce an anti-EBNA-1 antibody response. Specifically, we used an EBNA-1-specific enzyme-linked immunoassay to measure anti-EBNA-1 antibodies in mouse sera from four groups of four mice each: (a) mice killed 3 weeks after a single CLDC-based intravenous injection of CMV-luc-FR-CMV-EBNA-1, (b) mice killed 3 weeks after a CLDC-based repeated intravenous injection of CMV-luc-FR-CMV-EBNA-1,
The potential statistical significance of differences between groups was assessed using a two-sided, non-paired Student’s t test. B, groups of four female 25-g ICR mice were injected individually with 960 nmol of DOTIM/cholesterol MLVs complexed to 30 μg of CMV-luc-2 plus 30 μg of CMV-EBNA-1 and killed 1 day (d) or 1 week (w) after injection or to 30 μg of CMV-luc-2 plus 30 μg of CMV-CAT and killed 8 days after injection. The potential statistical significance of differences between groups was assessed using a two-sided, non-paired Student’s t test. iv, intravenous. c., control.

(c) mice killed 3 weeks after a single CLDC-based intravenous injection of CMV-luc-FR-2 only, and (d) un.injected control mice. We found that only three of the eight mice (two of four mice injected twice and one of four mice injected once) injected with the EBNA-1 gene developed weak (positive only at a 1:10 dilution) but detectable anti-EBNA-1 titers. Antibody levels were undetectable in the other five mice treated either once or twice with the CMV-luc-FR-CMV-EBNA-1 plasmid and did not significantly differ from levels in either CMV-luc-2 (mock)-treated or untreated control mice. Luciferase activity in mice with detectable anti-EBNA-1 antibody responses (6.9 ± 1.6 ng of luciferase/mg of tissue protein) did not differ significantly from that in mice without EBNA-1 antibody (8.6 ± 1.6 ng). Overall, we found that the presence of anti-EBNA-1 antibody responses did not appear either to limit the re-expression of this EBV-based plasmid vector system or to correlate with the level of luciferase activity produced by these vectors.

Pulmonary Cell Types That Express Delivered Genes for Prolonged Periods—To determine which cell types in mouse lungs are responsible for maintaining prolonged expression from EBV-based vectors, we used specific immunohistochemical assays to detect EBNA-1 and human p53 gene expression, respectively, both 24 h and 3 weeks after a single intravenous injection of cationic liposomes complexed to an expression plasmid containing CMV-human p53-FR-CMV-EBNA-1. Approximately 5–10% of all lung cells stained positively for EBNA-1 24 h after injection (Fig. 4A, panel a). Double antibody labeling techniques demonstrated that EBNA-1 was expressed in two cell types, vascular endothelial cells and alveolar epithelial lining cells (Fig. 4). Specifically, we used co-staining for anti-EBNA-1 and anti-factor VIII antibodies to show that lung endothelial cells were transfected and co-staining for anti-EBNA-1 and AE1/AE3 (an anti-cytokeratin antibody) antibodies to show that lung epithelial cells were also transfected (Fig. 4). Both vascular endothelial cells (24, 25) and alveolar epithelial cells (9, 41) have previously been shown to be transfected following intravenous injection of CLDCs in mice. The number of EBNA-1-positive lung cells fell ~100-fold over the 3-week period (Fig. 4A, panels f–h), confirming the localization of EBNA-1 expression to these two cell types. Neither EBNA-1 nor p53 expression was detectable in either mock-treated or untreated control mice (Fig. 4A, panel d; and data not shown). Compared with CLDC-based intravenous injection of EBNA-1 in an FR-containing plasmid (Fig. 4A, panels a–d), EBNA-1 immunoreactivity could still be detected in the lungs of mice 3 weeks after CLDC-based intravenous injection of CMV-EBNA-1 lacking FR, but at substantially reduced levels (data not shown). p53 immunoreactivity was still present 3 weeks post-injection in mice receiving CLDCs containing CMV-p53-FR-CMV-EBNA-1 (Fig. 4A, panel h), but was not detected in mice injected 3 weeks earlier with the CMV-p53-FR vector that lacked EBNA-1 plus FR (data not shown). Unlike human p53 protein, which has a very short half-life (42), EBNA-1 protein is quite stable (43, 44). Thus, the use of this EBV-based single plasmid vector system significantly prolonged the duration of expression of the p53 gene as well as of the EBNA-1 gene in mice.
Plasmids Containing Intact EBV oriP Do Not Replicate in the Presence of EBNA-1 in Mice—Previous studies have shown that oriP-containing plasmids are not stably replicated in the presence of EBNA-1 in rodent cell lines (17). However, replication of oriP-based plasmids in a subset of rodent cell lines tested has recently been reported (46, 58). Therefore, to determine whether EBV-based plasmids containing intact oriP could replicate in primary murine lung tissue, mice were injected intravenously with 20 µg each of oriP-BamHI C-Luc, oriP+, and either CMV-EBNA-1 or CMV-luc. After 14 days, the mice were killed, and low molecular weight DNA was isolated from lung tissue. The data in Table I indicate that although both oriP-BamHI C-Luc and oriP+ DNAs were present in mouse lungs 14 days after intravenous injection, neither plasmid was detectably replicated in either the presence or absence of EBNA-1. In contrast, oriP-BamHI C-Luc was efficiently replicated in the presence of EBNA-1 in human PPC-1 cells (Table I). However, oriP-BamHI C-Luc was not detectably replicated in the absence of EBNA-1 at 96 h post-transfection in PPC-1 cells (Table I). These results indicate that EBV-based plasmids containing intact oriP do not detectably replicate in primary murine tissue in either the presence or absence of EBNA-1. Previously, a human papovavirus-based expression plasmid has been shown to replicate in mouse lungs 2 weeks after a CLDC-based intravenous injection in mice (9), demonstrating that such replication is possible if appropriate sequences are present.

A Single Intravenous Co-injection of hG-CSF Plus FR- and EBNA-1-containing Plasmids Produces Therapeutic hG-CSF—We then assessed whether the ability of this EBV-based system to prolong gene expression in vivo could increase the therapeutic activity of a therapeutically important gene in mice. We tested the effects of the EBV-based two-plasmid system on both the duration of hG-CSF gene expression and its ability to increase the number of circulating white blood counts over time since recombinant hG-CSF is used in human patients to increase white blood counts in neutropenic patients post-chemotherapy (47). We measured the level of hG-CSF in mouse serum by enzyme-linked immunosorbent assay following intravenous injection of CLDCs containing CMV-hG-CSF (30) or p4402, CMV-hG-CSF-FR, co-injected with CMV-EBNA-1. ICR mice injected with CMV-hG-CSF-FR plus CMV-EBNA-1 expressed 4261 ± 457 pg/ml hG-CSF in mouse serum at day 1, 41, 31, and 62 after injection, respectively. In contrast, mice injected with CMV-hG-CSF lacking FR plus CMV-EBNA-1 expressed 5274 ± 3333 pg/ml hG-CSF protein in their serum at day 1, but serum hG-CSF was not detectable (<20 pg/ml) at 3 or 7 days following injection (Table II).

Previously, serum levels of hG-CSF above 100 pg/ml have been shown to maintain absolute neutrophil counts (ANCs) at stably elevated levels over a period of several months (48). Therefore, we determined both the ANCs/mm³ of whole blood and the percentage of band (immature neutrophil) forms in untreated mice and in mice that received a single intravenous injection of CLDCs containing either CMV-hG-CSF-FR or CMV-luc-FR plus CMV-EBNA-1 2 or 8 weeks earlier. Mice receiving CMV-hG-CSF-FR plus CMV-EBNA-1 showed an ~4–5-fold elevation in ANCs at both 2 and 8 weeks after injection compared with ANCs in mice receiving either CMV-luc-FR together with CMV-EBNA-1 or no treatment (Table II). Furthermore, 2–4% of the neutrophils present were band forms in mice receiving CMV-hG-CSF-FR plus CMV-EBNA-1, whereas band forms were not detected in either CMV-luc-FR-2-treated or untreated mice (p < 0.005 for CMV-hG-CSF-FR-treated mice versus either CMV-luc-FR-treated or untreated mice for both ANCs and the percentage of band forms) (Table II).

Therefore, even the 4% of peak hG-CSF levels still present at 8 weeks was sufficient to maintain high ANCs and therefore the full biologic activity of hG-CSF. Conversely, conventional plasmid vectors lacking EBNA-1 plus FR were unable to increase either ANCs or immature neutrophils at any time point following intravenous injection. The failure of the conventional plasmid vector to produce any biologic activity was due exclusively to the transient expression of the hG-CSF gene since
peak levels produced by both conventional and EBV-based vectors were comparable (Table II). Moreover, in preliminary studies, intravenous injection of CLDCs containing CMV-hG-CSF-FR plus CMV-EBNA-1 into female New Zealand White rabbits (~3.0 kg in weight) significantly increased their ANC for >1 week, whereas ANCs were not increased in rabbits receiving intravenous CLDCs containing CMV-hG-CSF plus CMV-EBNA-1, similar to results obtained in mice (Table II and data not shown).

In addition, we tested whether pure DOTMA liposomes, because they are more efficient than DOTIM/cholesterol for intravenous gene delivery (49), could further prolong therapeutic levels of hG-CSF gene expression following intravenous CLDC-based co-injection in this EBV-based vector system. We found that a single intravenous co-injection of CMV-hG-CSF-FR plus CMV-EBNA-1 complexed to pure DOTMA liposomes produced serum hG-CSF levels of 1876 ± 82 pg/ml 31 days later. This is >4-fold higher than that produced 31 days after a single intravenous co-injection of the same dose in this vector system complexed to DOTIM/cholesterol liposomes (Table II).

The EBNA-1-FR-based Vector System Functions in Other Mouse Strains—Finally, to determine whether this EBV-based vector system functions to prolong gene expression in mouse strains other than ICR, we also assessed the effects of co-injecting CMV-luc-FR-2 (p4379) plus CMV-EBNA-1 (p4395) on the duration of luciferase gene expression in groups of four BALB/c and C57Bl6 mice. A single CLDC-based intravenous co-injection of CMV-luc-FR-2 plus CMV-EBNA-1 produced 6.0 ± 0.9 ng of luciferase/mg of tissue protein in the lungs of BALB/c mice killed 4 weeks later and 1.6 ± 0.8 ng of luciferase/mg of tissue protein in the lungs of C57Bl6 mice killed 4 weeks later. These levels were significantly higher than the luciferase activity produced in the lungs (0.2 ± 0.1 ng of luciferase/mg of tissue protein; p < 0.005) of either BALB/c or C57Bl6 mice killed 4 weeks after intravenous injection of CLDCs containing CMV-luc-FR-2 alone (in the absence of CMV-EBNA-1) or of un.injected mice. In addition, we measured anti-EBNA-1 antibody levels in these BALB/c and C57Bl6 mice injected with these EBV-based plasmid vectors. Anti-EBNA-1 antibody responses were present at a 1:10 dilution in two of five BALB/c mice intravenously injected 3 weeks earlier with CLDCs containing CMV-EBNA-1, whereas none of the C57Bl6 mice injected with this vector showed detectable anti-EBNA-1 antibodies. As seen with ICR mice, the presence of anti-EBNA-1 antibodies in individual BALB/c and C57Bl6 mice did not appear to correlate with the levels of luciferase activity produced. Overall, the presence of EBNA-1 plus FR in CLDC-injected plasmid vectors significantly prolonged gene expression in each of the three different mouse strains we tested.

**DISCUSSION**

In summary, we observed that CLDC-based systemic delivery of this EBV-based vector system can (a) produce therapeutically relevant levels of expression of biologically important genes at least 2 months following a single intravenous injection; (b) then efficiently re-express delivered genes, despite repeated intravenous re-injection into fully immunocompetent animals; and (c) function without requiring prolonged overexpression of a potentially transforming viral DNA-binding protein. This approach, non-replicating EBV-based Plasmids

**FIG. 4.** A, immunohistochemical staining for EBNA-1 (panels a–d) and p53 (panels e–h). Panel a, photomicrograph (magnification × 10) of anti-EBNA antibody-stained mouse lung 24 h post-intravenous injection of CLDCs containing CMV-p53-FR-CMV-EBNA-1. Black nuclei are positive for EBNA-1 expression. Panel b, photomicrograph (magnification × 10) of panel a showing elongated endothelial nuclei and rounded epithelial nuclei that are positive for EBNA-1. Panel c, photomicrograph (magnification × 60) of panel a showing a central small vessel (arrowhead) with EBNA-1-immunoreactive endothelial cells and refractile-appearing red blood cells. Panel d, photomicrograph (magnification × 60) of anti-EBNA antibody-stained mouse lung 3 weeks post-intravenous injection of CLDCs containing CMV-p53-FR-CMV-EBNA-1 showing EBNA-1-immunoreactive nuclei and routinely negative respiratory epithelium (arrowhead). Panel e, photomicrograph (magnification × 20) of CMV-luc-injected control mouse lung showing no immunoreactivity for EBNA-1. A similar lack of immunoreactivity for human p53 was observed on control slides. Panel f, photomicrograph (magnification × 60) of CMV-p53-FR-CMV-EBNA-1-injected mouse lung 24 h post-injection. Cells staining positively for p53 are predominantly endothelial, with small elongated nuclei. Panel g, photomicrograph (magnification × 60) of CMV-p53-FR-CMV-EBNA-1-injected mouse lung 24 h post-injection showing both epithelial and endothelial nuclei positive for p53 expression. Panel h, photomicrograph (magnification × 60) of CMV-p53-FR-CMV-EBNA-1-injected mouse lung 3 weeks post-injection showing substantially reduced numbers of clearly p53-positive cells (predominantly endothelial). A, immunohistochemical double label staining for EBNA-1 and either factor VIII or AE1/AE3 (cytokeratin). Panel a, photomicrograph (magnification × 10; inset, magnification × 60) of an EBNA-1-positive cell (brown nucleus) in a cell negative for endothelial (anti-factor VIII/von Willebrand factor) staining (no red in cytoplasm; tip of arrowhead, see high-power inset). Note endothelium-positive staining in nearby vessel (broad end of arrowhead). Panel b, photomicrograph (magnification × 10; inset, magnification × 60) showing double-staining EBNA-1-positive and factor VIII-positive endothelial cells (arrowheads and top and bottom positive cells in inset). Panel c, photomicrograph (magnification × 20; inset, magnification × 40) of double-staining EBNA-1-positive nuclei (black and brown; see arrowheads) with cytokeratin-positive cytoplasm of epithelial cells. Panel d, photomicrograph (magnification × 20; inset, magnification × 60) of elongated EBNA-1-positive nuclei with cytokeratin-negative cytoplasm of endothelial cells (arrowhead and inset).
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### Table I

| Cells          | orIP-digested effector | orIP-undigested | orIP-10^5 cells |
|----------------|------------------------|----------------|----------------|
| Lung EBNA-1    | 8600 ± 6400           | 2.2 ± 0.96     | 3.7 ± 1.04     |
| Lung CMV-luc   | 16,000 ± 6400         | 2.6 ± 3.8      | 3.7 ± 1.3      |
| PPC-1 EBNA-1   | 2.1 ± 0.34 x 10^5     | NT            | NT             |
| PPC-1 CMV-luc  | <4830                 | NT            | NT             |

*Twenty μg each of orIP-BamHI C-Luc, orIP- and effector DNA encoding EBNA-1 or CMV-luc were co-complexed to DOTIM cholesterol liposomes and injected into the tail vein of mice or transfected into PPC-1 cells (32). The amount of plasmid DNA was measured by quantitative competitive polymerase chain reaction as described previously (33), except that the primers used in these experiments were end-labeled with [γ-32P]ATP prior to performing the polymerase chain reaction (33). A sample was considered to have detectable EBNA-1 if the signal was greater than 2.0% of the control.

**TABLE II**

hG-CSF protein levels that significantly increase ANC and band counts a maintained in the serum of ICR mice for prolonged periods following hG-CSF gene delivery via intravenous injection of CLDC containing CMV-hG-CSF-FR plus CMV-EBNA-1

| Vector (plus CMV-EBNA-1)** | Days post-injection | Serum hG-CSF levels | Increase in ANC vs untreated controls | Bands | p value: hG-CSF levels vs untreated controls |
|-----------------------------|---------------------|---------------------|----------------------------------------|-------|-------------------------------------------|
| CMV-hG-CSF-FR               | 1                   | 4861 ± 2606         | NT                                     | NT    | <0.05                                      |
| CMV-hG-CSF-FR               | 14                  | 636 ± 45            | 477 ± 55d                             | 2.0 ± 1.1d | <0.05                                      |
| CMV-hG-CSF-FR               | 31                  | 457 ± 86            | NT                                     | NT    | <0.05                                      |
| CMV-hG-CSF-FR               | 62                  | 187 ± 74            | 417 ± 64d                             | 3.0 ± 2.0d | <0.05                                      |
| CMV-hG-CSF                 | 3                   | <20                 | 0                                      | 0     | <0.4                                       |
| CMV-hG-CSF                 | 7                   | <20                 | 0                                      | 0     | <0.4                                       |
| CMV-luc-FR-2               | 14                  | <20                 | 0                                      | 0     | <0.4                                       |
| CMV-luc-FR-2               | 62                  | <20                 | 0                                      | 0     | <0.4                                       |
| No DNA (untreated)          | 0                   | <20                 | 0                                      | 0     | <0.05                                      |

* Nine-hundred sixty nmol of DOTIM/cholesterol MLVs complexed to 30 μg of the indicated vector plus 30 μg of CMV-EBNA-1 per mouse were injected intravenously into groups of four ICR mice on day 0. Mice were killed and bled at the days indicated. hG-CSF levels (mean ± S.E.) were measured as previously described (30).

**Total white blood cell counts were determined with a hemocytometer using EDTA-anticoagulated blood diluted in a Unopette white cell test system. Differential counts were performed by an individual blinded to the experimental design using blood smears stained with Diff-Quik.

***p < 0.005 compared with either ANC or band counts in untreated control mice by a two-sided Student’s t test.

This hypothesis (50). Retention of FR-containing plasmids by cellular chromosomes may be required to prolong the expression of delivered genes even in non-dividing cells since episomal plasmids lacking such retention sequences may be more efficiently degraded or excised by these cells.

Interestingly, a similar level of both orIP-containing as well as orIP-lacking DNA was observed in the presence or absence of EBNA-1 at 14 days post-injection (Table I). Similarly, the levels of hG-CSF in the serum of ICR mice when expressed from a vector either containing or lacking FR were comparable in the presence of EBNA-1 at 24 h post-injection. In contrast, by day 3 post-injection, hG-CSF expressed from a vector lacking FR was undetectable, whereas >9-fold more hG-CSF was expressed from a vector containing FR even 62 days later (Table II). Thus, EBNA-1 is playing an active role in enhancing gene expression in this system either through recruiting, chaperoning, or retaining the transfected DNA within the appropriate compartment of the nucleus for efficient expression or through functioning as a transcriptional activator or both.

EBNA-1 itself is insufficient in the context of EBV to immortalize primary human B lymphocytes in vitro in the absence of the latent viral proteins EBNA-2 (52), EBNA-3A (53), EBNA-3C (53), and LMP-1 (54). However, mice transgenic for EBNA-1 have been reported to develop B cell tumors (12). Furthermore, human papovavirus- or SV40-based replicating
The ability of transiently expressed EBNA-1 to mediate prolonged expression from co-injected FR-containing plasmids is consistent with the overall stability of the EBNA-1 protein. The EBNA-1 protein does not detectably degrade over a 20 h time course in pulse-chase experiments using a vaccinia virus system expressing EBNA-1 in CV-1 cells (43) or using an EBV-course in pulse-chase experiments using a vaccinia virus system expressing EBNA-1 in CV-1 cells (43) or using an EBV-positive lymphoblastoid cell line, GM2783 (44). The stability of EBNA-1 is partly due to its ability to evade degradation by the ubiquitin-proteasome pathway by virtue of its Gly-Gly-Ala repeats. However, even a mutant form of EBNA-1 lacking the Gly-Gly-Ala repeats has a half-life of ~18 h in cells (43), reflecting the resistance of EBNA-1 to degradation by proteasome-independent pathways as well. Thus, it is likely that once EBNA-1 is expressed (even transiently) in the target tissue, some of the expressed protein may remain present and functional for prolonged periods.

The ability of the EBNA-1 plasmid to mediate prolonged expression of FR-containing plasmids, even after repeated intravenous re-injection of CMV-EBNA-1 in fully immunocompetent mice (Fig. 2), may in part be explained by the ability of EBNA-1 to limit the generation of EBNA-1-specific cytotoxic T lymphocyte responses both in humans and in mice (45, 55, 56). This ability is mediated by the Gly-Gly-Ala repeats within EBNA-1 that generate a cis-acting inhibitory signal that appears to interfere with antigen processing and major histocompatibility complex class I-restricted presentation of EBNA-1 (45, 55, 56). In contrast to this EBV-based plasmid approach, which can prolong the duration of gene expression at therapeutic levels for a period of several months and then efficiently re-express the gene following repeated re-injection into immunocompetent animals, several recombinant viral vectors have been shown to produce long-term gene expression for ≥6 months following a single in vivo administration (1, 2). However, the induction of host immune responses directed against expressed viral gene products can limit or prevent re-expression of such viral vectors in immunocompetent animals (3, 4, 6).

More recent studies indicate that viral vectors can induce cytotoxic T lymphocyte-mediated immune responses, even in the absence of either viral replication or de novo protein synthesis (5). Furthermore, unlike integrating AAV retro- and lentiviral vectors that can produceinsertional mutagenesis, our EBV-based vector appears to remain episomal (Table I), thus reducing or obviating this potential risk.

Our results indicate that this EBV-based two-plasmid vector system can significantly improve the biologic and therapeutic activity of hG-CSF following CLDC-based intravenous delivery of the angiostatin gene in mice bearing either metastatic melanoma or metastatic mammary carcinoma compared with the angiostatin gene delivered in similar vectors lacking FR plus EBNA-1 (Ref. 49 and data not shown). Thus, the ability of EBV-based vectors to prolong intravenous gene expression and then to efficiently re-express the gene may increase the efficacy of those genes whose full activity depends on extending their expression in vivo.

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