Allogeneic Transplantation Induces Expression of Cytomegalovirus Immediate-Early Genes In Vivo: a Model for Reactivation from Latency

MARY HUMMEL,1,2 ZHENG ZHANG,1 SHIXIAN YAN,1 ISABELLE DEPLAEN,3 PIYUSH GOLIA,1 THOMAS VARGHESE,1 GAIL THOMAS,1 AND MICHAEL I. ABECASSIS*1,4

Department of Surgery, Division of Organ Transplantation,1 Department of Microbiology and Immunology,2 and Department of Pediatrics,3 Northwestern University Medical School, Chicago, Illinois 60611

Received 20 November 2000/Accepted 14 February 2001

Reactivation of cytomegalovirus (CMV) from latency is a frequent complication of organ transplantation, and the molecular mechanism by which this occurs is unknown. Previous studies have shown that allogeneic stimulation induces reactivation of human CMV (HCMV) in vitro (64). We find that transplantation of vascularized allogeneic kidneys induces murine CMV (MCMV) and HCMV immediate-early (ie) gene expression. This induction is accompanied by increased expression of transcripts encoding inflammatory cytokines, including tumor necrosis factor (TNF), interleukin-2, and gamma interferon, and by activation of NF-κB. TNF alone can substitute for allogeneic transplantation in inducing HCMV and MCMV ie gene expression in some tissues. Our studies suggest that reactivation is a multistep process which is initiated by factors that induce ie gene expression, including TNF and NF-κB. Allogeneic transplantation combined with immunosuppression may be required to achieve complete reactivation in vivo.

Cytomegalovirus (CMV) is a ubiquitous herpesvirus which infects 60 to 90% of adults (reviewed in reference 50). Primary infection in immunocompetent hosts is self-limiting and is generally not associated with significant morbidity or mortality. Replicating virus is eventually cleared by the host immune response, but the virus establishes a lifelong latent or persistent infection. CD34+ hematopoietic progenitor cells and cells of the monocyte/macrophage lineage have been identified as sites of latent human CMV (HCMV) infection (27, 49, 62, 70). Reactivation of latent virus accompanied by asymptomatic viral shedding can occasionally occur in healthy, seropositive individuals. In contrast, reactivation of latent virus is frequently observed in immunocompromised individuals, such as transplant recipients and AIDS patients, and, despite the development of effective antiviral drugs, remains a significant cause of morbidity and mortality.

Due to the species specificity of HCMV, many investigators have turned to murine CMV (MCMV) as a model for the study of CMV latency and reactivation. MCMV is similar to HCMV with respect to genome organization, pathogenesis, and ability to establish latent infection and to reactivate. It has been previously demonstrated in mice latently infected with MCMV that latent viral DNA is present in bone marrow cells, in alveolar macrophages, and in endothelial cells in the kidney, liver, heart, and spleen (34). Thus, endothelial cells may represent an important site of latency for HCMV as well.

Latency has been defined operationally as the inability to detect infectious virus despite the presence of viral DNA. However, it has not been clear whether lack of virus production is due to establishment of a true latent state, in which gene products associated with lytic replication are not expressed, or whether small numbers of permissively infected cells are usually present with spread of infection blocked by the immune system. Reactivation in the former case would be due to a change in the transcriptional program of the latently infected cell, while in the latter, reactivation would be the result of failure of the immune system to remove productively infected cells (36). A number of studies have investigated expression of RNAs associated with productive infection in tissues of latently MCMV-infected mice or in latently HCMV-infected cells. Some studies have reported that MCMV immediate-early protein-1 (IE1) transcripts are not detectable in tissues of latently infected mice (34), while others have reported that they are detectable in some organs (5, 30, 37, 77, 78). It is not clear whether this discrepancy is due to differences in sensitivity of detection, to methods used for infection, or to spontaneous reactivation of the virus. The most definitive study done to date suggests that although expression of IE1 transcripts is detectable in some regions of organs from latently infected mice, it is not detectable in all regions which carry latent viral DNA and “is not a feature inherent to murine CMV latency but rather reflects foci of primordial reactivation” (37). HCMV IE1 transcripts initiated at a novel latency-specific promoter and antisense IE1 transcripts have been identified in a small percentage of granulocyte-macrophage progenitor cells infected in vitro and in bone marrow cells of healthy, seropositive individuals (27, 35, 63), but their role in the CMV life cycle is unclear (74). Others have reported that HCMV IE1 transcripts are not detectable in latently infected CD34+ hematopoietic progenitor cells (49).

Studies with animal models of CMV have shown that reactivation can be induced by immunosuppressive therapies alone, such as total body irradiation or administration of cytotoxic drugs like cyclophosphamide and azathioprine or by immunodepletion of T cells or T-cell subsets (5, 12, 47, 48, 53). One
recent study concluded that, unlike the case with other herpesviruses, CMV latency is maintained primarily as a result of immune surveillance rather than by transcriptional control in latently infected cells (53). However, the immunosuppressive regimes employed in these studies result in high levels of cell death and release of inflammatory cytokines, which may themselves play a role in reactivation (7, 16, 19, 23, 25, 26, 55). In clinical settings, reactivation of CMV is frequently associated with rejection of the transplanted organ or with other conditions accompanied by high levels of inflammatory cytokines, such as graft-versus-host disease, cirrhosis, and sepsis (19, 46, 51). With less cytotoxic immunosuppressive regimens, such as the use of cyclosporine, the frequency of reactivation is much lower than that observed with earlier therapies, both in animals and in clinical settings (12, 31, 59).

Allogeneic stimulation has also been proposed as a factor in inducing reactivation of latent virus (64). The role of allogeneic stimulation in inducing CMV reactivation has been studied in animal models by using organ transplantation, tissue implantation, blood transfusion, or cell transfer. Most of these studies demonstrate that allogeneic stimulation plays an important role in the reactivation of latent CMV (11, 13, 21, 61, 76), although others have found that in the absence of immunosuppression, there is a higher frequency of reactivation with syngeneic cell transfer or tissue implantation (29, 47). More recently, reactivation of latent HCMV has been achieved by allogeneic stimulation of peripheral blood mononuclear cells in vitro (64), suggesting that allogeneic stimulation may indeed be an important factor in inducing reactivation in vivo. None of these studies has addressed the mechanism by which allostimulation might lead to reactivation of CMV. Reactivation of HCMV has also been achieved by cytokine treatment of experimentally infected granulocyte-macrophage progenitor cells in vitro (27). Tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), and, to a lesser extent, granulocyte-macrophage colony-stimulating factor and interleukin-4 (IL-4) were all found to induce reactivation in this system. The mechanism by which this occurred was not explored.

In view of the conflicting data on viral gene expression in latently infected cells and on the role of immune suppression and allogeneic stimulation in inducing CMV reactivation, we have further investigated the effect of allogeneic and syngeneic transplantation in inducing expression of genes required for lytic replication of MCMV. These studies were performed in the absence of immunosuppression to study the role of allostimulation alone without the potential confounding effects of cytokine release due to immunosuppression. We have compared the level of viral gene expression in transplanted donor kidneys with that in the contralateral kidneys removed at the time of transplant in order to determine the level of viral gene expression before and after transplantation. Thus, even if some ie gene expression is detectable in the latently infected animals, the specific effects of transplantation on ie gene expression can be determined. We find that allogeneic transplantation induces expression of the MCMV and HCMV ie genes. This induction is accompanied by increased expression of transcripts encoding inflammatory cytokines, including TNF, IL-2, and IFN-γ, suggesting that these cytokines could mediate induction of viral gene expression. In addition, we have investigated the ability of TNF to induce viral gene expression directly by injecting TNF into latently infected mice. Our studies suggest that reactivation is a multistep process which is initiated by factors induced as a result of allogeneic transplantation. TNF activates transcription factors which regulate expression of the ie1 gene, including NF-kB, and TNF alone is able to induce ie1 gene expression in some tissues. Given the fact that ie1 gene expression is very low or is not detectable in tissues from latently infected mice (34, 37) and that expression of this gene is required to initiate productive viral infection in vitro, induction of ie1 gene expression is likely to be a key step in viral reactivation. Our results suggest a mechanism by which this might occur.

MATERIALS AND METHODS

Animals. Three-week-old female, specific-pathogen-free BALB/c mice and adult male BALB/c (H-2b), C57BL/6 (H-2d), and C3H/HeN (H-2k) mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Breeding pairs of MIEP-lacZ transgenic mice from the TG11B line (4) carrying the β-galactosidase gene under the control of the HCMV ie promoter were obtained from Jay Nelson at Oregon Health Sciences University. Mice were maintained in isolation cages and were fed and watered ad libitum. This study protocol was reviewed and approved by the Northwestern University Institutional Animal Care and Use Committee.

Virus infection and establishment of MCMV latency. Three- to 4-week-old mice were injected intraperitoneally with 10⁷ PFU of MCMV (Smith strain) and maintained for 6 months to establish latent infection as previously described (34).

Transplants and organ processing. Mouse kidney transplants were performed as previously described (80). The right donor kidney from latently infected mice was removed for use as a control, and the left donor kidney was transplanted into syngeneic BALB/c (H-2b) or allogeneic C57BL/6 (H-2d) adult males and removed at 1, 2, 5, 8, or 15 days after transplant. Kidneys from MIEP-lacZ transgenic mice were transplanted into allogeneic C3H/HeN (H-2k) mice and were removed 2 days after transplant. All organs were frozen in liquid nitrogen immediately after removal.

Cytokine injections. For RNA analysis, mice were injected intraperitoneally with 10 µg of TNF (R & D Systems, Minneapolis, Minn.) or in the tail vein with 2.5 µg of TNF and were sacrificed at various times after injection. No difference in MCMV RNA expression was observed with intraperitoneal or intravenous injections. For gel shifts or analysis of β-galactosidase expression, mice were injected intravenously with 2.5 µg of TNF and were sacrificed after 2 or 24 h, respectively.

RT-PCR analysis. Mice were anesthetized with Metofane (Schering-Plough) and were sacrificed by cervical dislocation. For RNA extraction, frozen tissues were sonicated in TriReagent (Molecular Systems, Cincinnati, Ohio) to disrupt the tissue and the RNA was purified according to the directions of the manufacturer. Reverse transcriptase PCR (RT-PCRs) were performed with an RNA PCR kit (Perkin-Elmer Cetus) according to the directions of the manufacturer. RNA (1.5 µg) was reverse transcribed using random hexamer primers, and the cDNA was amplified in 40 cycles of 94°C, 30 s; 58°C, 30 s; and 72°C, 30 s, followed by a 7-min incubation at 72°C. PCR products were electrophoretically separated on 1.5% agarose gels, transferred to nitrocellulose, and hybridized to oligonucleotide probes at 42°C for 2 h. Probes were labeled with fluorescein-dUTP and terminal transferase using an enhanced chemiluminescence 3’-oligo-labeled nucleotide kit (Amersham) and were detected according to the directions of the manufacturer. The following primers and probes were used to amplify and detect RT-PCR products: for ie1, primer 1, primer 2 (1), 179 bp, probe, CH15 (30); for ie3, CH17 (30), IE3-RT/R (38), 229 bp, probe, CH15 (30); for E-1, forward, reverse (5), 266 bp, probe, GCCAGCCGGACCGGAGGCA GAGGCGGCCCTCGAGTACAAGGAG; for IE1, forward, reverse (5), 400 bp, probe, AAGCGAAAACCATGTCTCTCGGTCCGCTACGAGGG; for TNF, sens, antisens (33), 678 bp, probe, CAGTAGACAGAAGAGGTGTGGGCC CCTGCCACAAGCAGG; for IL-2, 5’ gene, 5’ 3’ gene, 3’ 5’ gene, 247 bp, probe, GAGCTCTGAGCGAGTGTAGAAAGAAGAAGAgG; for IFN-γ, sens (33), 401 bp, probe, GCCAGCATTACGTCTCTCTCGTCCAGTCTTG AAGTC; for IL-1β, 5’ AGGCTTCACCACATGAGACGACGACGACGACG; for 3’ CTAAGAGCGCAGCTTGTGTGATA; for β-actin, 5’ gene, 5’ 3’ 3’ gene, 260 bp, probe, GAGGCTACATGCATGCTGCC; for MCMV TGCGTTG and 3’ ATCTGCTGGAGAAGTGGCATGAG, 453 bp.

Analysis of lacZ transgene expression. Histochemical staining of tissue sections from MIEP-lacZ transgenic mice for β-galactosidase activity was performed as previously described (4). β-Galactosidase activity in tissues from
MIEP-lacZ transgenic mice was assayed with a Galacto-Star chemiluminescent reporter gene assay system (Tropix PE Biosystems, Bedford, Mass.) in triplicate as described by the manufacturer and was quantitated with a Monolight 2010 luminometer. Protein concentration in the extract was determined by Bio-Rad protein assay, and values were expressed as average light units of β-galactosidase per nanogram of protein.

Electrophoretic mobility shift assay. Nuclear extracts were prepared from renal and lung tissue as previously described (18), assayed for protein concentration (9), and stored at −80°C. NF-κB and AP-1 consensus oligonucleotides (Promega Co., Madison, Wis.) were labeled with [γ-32P]ATP (3,000 Ci/mmol, 10 mCi/ml; Amersham, Arlington Heights, Ill.) using T4 polynucleotide kinase. Extract (3.5 μg) in 10 μl was precultivated with 4 μl of gel shift binding buffer (18) at 25°C for 15 min and was then incubated with 1 μl of probe for 20 min at 25°C and analyzed as previously described (17, 18). Competition experiments were performed by incubation of extracts with a 100-fold excess of unlabeled oligonucleotide containing consensus or mutant NF-κB binding sites (Santa Cruz Biotechnology, Santa Cruz, Calif.) for 20 min prior to addition of the probe. Supershift experiments were performed by incubating extracts, oligonucleotides, and 1 μg of p50, p65, p52, RelB, or c-Rel antibody (Santa Cruz Biotechnology) for 20 min prior to electrophoresis.

RESULTS

Allogeneic transplantation induces ie1 gene expression. In order to investigate the role of allogeneic stimulation in inducing MCMV lytic cycle gene expression, kidneys from latently infected BALB/c (H-2d) mice were transplanted into uninfected syngeneic recipients and were removed 1, 2, or 5 days after transplant (lanes 5, 7, and 9, respectively). RNAs from transplanted kidneys (T) or contralateral control (C) kidneys or from productively infected murine embryo fibroblasts (lanes 1 and 2) were analyzed for MCMV IE1 RNA expression by RT-PCR. Transplanted and contralateral control kidneys are indicated by brackets. Lane 3, β-Actin RNA was analyzed as a positive control.

and this induction was due to the allogeneic response rather than ischemia/reperfusion injury.

RNAs isolated from control and transplanted kidneys were also examined for expression of IE3, an alternatively spliced transcript initiated at the ie1 promoter; E-1, an early gene product; and gB, a late gene product. No expression of these transcripts was observed, indicating that although the initial phase of viral replication was induced by allogeneic stimulation, full reactivation with lytic replication was not induced (data not shown). The lack of expression of IE1 at later times after transplant and the lack of expression of other genes associated with productive infection are likely to be due to destruction of cells expressing foreign antigens by immune surveillance in these immunocompetent recipient.

NF-κB is activated by allogeneic transplantation. Regulation of HCMV and MCMV ie gene expression is controlled by the CMV ie promoter/enhancer (8, 20, 68, 72). Both the MCMV and HCMV ie promoter/enhancer regions contain multiple binding sites for NF-κB, as well as other transcription factors (Fig. 2). The NF-κB sites and the ATF(CREB) sites have been shown to be important in regulation of the HCMV promoter (32, 41, 60, 68). The factors important in regulation of the MCMV promoter have not been studied. In order to investigate the effect of transplantation on activation of transcription factors that are likely to be important in controlling MCMV ie gene expression, we performed gel shift analysis on nuclear extracts of kidneys of latently infected mice transplanted into syngeneic or allogeneic recipients (Fig. 3). The contralateral kidney from each donor was removed at the time of transplant and was analyzed as a control. Two NF-κB complexes were observed in extracts of control and transplanted kidneys. Both complexes bound specifically to the consensus NF-κB binding site, since an unlabeled NF-κB oligonucleotide competitively abolished binding, but an oligonucleotide with a mutated NF-κB binding site did not (data not shown). The more slowly migrating complex is a classical NF-κB complex, since it was supershifted by antibodies specific for the p65 and p50 subunits of NF-κB. The faster-migrating complex was not supershifted with antibodies to any of the known NF-κB subunits, including p65, p50, p52, c-Rel, and Bcl, and its composition is therefore unknown. A slight activation of NF-κB was observed in syngeneic transplants (Fig. 3, left panel, compare lanes 1 and 2 and lanes 5 and 6). This is likely to be due to ischemia/reperfusion injury, which has been

FIG. 1. Effect of transplantation on expression of MCMV IE1 RNA. Kidneys from latently infected mice were transplanted into allogeneic or syngeneic recipients and were removed 1, 2, or 5 days after transplant (lanes 5, 7, and 9, respectively). RNAs from transplanted kidneys (T) or contralateral control (C) kidneys or from productively infected murine embryo fibroblasts (lanes 1 and 2) were analyzed for MCMV IE1 RNA expression by RT-PCR. Transplanted and contralateral control kidneys are indicated by brackets. Lane 3, RNA from lung tissue of a latently infected mouse injected with TNF.

FIG. 2. Putative transcription factor binding sites in the MCMV and HCMV ie1 promoter/enhancer regions. Binding sites in the MCMV promoter/enhancer are based on analysis of the nucleotide sequence (20). Binding sites in the HCMV promoter/enhancer are based on previous analyses (41).
previously reported to activate NF-κB. However, much greater induction was observed at day 2 in the allogeneic transplants than in syngeneic transplants (Fig. 3, left panel, compare lanes 7 and 8 with lanes 5 and 6). This coincides with the peak of IE1 RNA expression previously observed in latently infected kidneys transplanted into allogeneic recipients (Fig. 1). Activation of AP-1 was observed in both allogeneic and syngeneic transplants and was therefore due to ischemia/reperfusion injury. Previous reports have demonstrated activation of AP-1 and NF-κB due to ischemia/reperfusion injury and activation of NF-κB due to allogeneic transplantation (10, 14, 81).

Expression of inflammatory cytokines is induced by allogeneic transplantation. NF-κB is activated by several different mediators of the immune and inflammatory response, including the proinflammatory cytokines IL-1 and TNF. Allogeneic stimulation results in activation of TH1 cells and release of inflammatory cytokines. We therefore analyzed RNAs from transplanted kidneys for expression of TNF, IL-2, IFN-γ, and IL-1 (Fig. 4). Expression of IL-1 was present in control kidneys and was not significantly induced in allogeneic or syngeneic transplants. In contrast, little or no expression of TNF, IL-2, and IFN-γ was observed in control kidneys. Expression of these cytokines was specifically induced after 24 h in allogeneic but not in syngeneic transplants. Induction of ie1 gene expression was observed 24 to 48 h after allogeneic transplantation. Thus, expression of these cytokines was induced prior to or coincident with induction of ie1 gene expression. These data are consistent with the hypothesis that inflammatory cytokines activate transcription factors which induce expression of the ie1 gene. TNF is known to induce activation of both NF-κB and AP-1 (42, 52, 65, 73), and TNF has been shown to induce expression of HCMV ie promoter-driven reporter constructs in vitro via activation of NF-κB (54, 67). These observations suggest that TNF could mediate induction of MCMV ie1 gene expression in vivo in response to allogeneic transplantation.

TNF induces MCMV ie1 gene expression in the lung. In order to test this hypothesis directly, latently infected mice were injected with recombinant murine TNF and analyzed for ie1 gene expression at various times after injection. Induction of IE1 RNA was consistently observed in lung tissue from latently infected mice 8 to 24 h after injection of TNF (Fig. 5), although the kinetics of induction varied somewhat. No expression of IE1 RNA was detected in the lung at 48 h after injection, and no induction of E-1 or gB transcripts was observed at any time point (data not shown). This is likely to be due to immune destruction of cells expressing IE1 protein in these immunocompetent mice with immunological memory of MCMV. Surprisingly, no induction of IE1 transcripts was observed in kidney, heart, liver, or spleen from latently infected mice injected with TNF.

Gel shift analysis of nuclear extracts of lung and kidney tissue from mice injected with TNF was performed to identify the transcription factors activated by TNF (Fig. 5). As was the
case with transplanted kidneys (Fig. 3), two complexes with NF-κB-binding activity were observed. Both complexes bound specifically to the consensus NF-κB binding site, since binding was abolished by excess unlabeled NF-κB oligonucleotide (Fig. 5B, lane 9) but not by a mutant NF-κB oligonucleotide (Fig. 5B, lane 8). Supershift analysis demonstrated that the more slowly migrating complex contained p65 and p50 (data not shown) subunits of NF-κB, while the more rapidly migrating complex was not recognized by antibodies specific for any NF-κB subunit, including p65, p50, p52, c-Rel, and RelB. Injection with TNF resulted in a statistically significant activation of NF-κB in both the lung and the kidney (Fig. 5B). The mobility of the NF-κB complex appeared to be slightly different in kidney and lung extracts, suggesting that the composition of the complex could be different. However, supershift analysis indicated no obvious differences in the compositions of the lung and kidney complexes (data not shown). In contrast, examination of AP-1 activation demonstrated that TNF activated AP-1 in the lung but not in the kidney (Fig. 5C). The mobility of this complex was significantly greater than that present in transplanted kidneys, suggesting that the composition of the AP-1 complex could differ between lung and kidney. Thus, our gel shift analysis indicates that induction of IE1 gene expression correlates with activation of both NF-κB and AP-1, suggesting that NF-κB and AP-1 may act cooperatively to induce MCMV ie1 gene expression. TNF can substitute for allogeneic transplan-

**FIG. 5.** Effect of TNF on MCMV ie1 gene expression and transcription factor activation in lung and kidney tissue of latently infected mice. (A) RT-PCR analysis of IE1 RNA expression in lungs of latently infected mice at 8, 16, 24, and 48 h after injection with TNF. RNA from productively infected murine embryo fibroblasts (Acute) was analyzed as a positive control. NT, no template. (B and C) Electrophoretic mobility shift assay analysis of activation of NF-κB and AP-1 in lung (L) and kidney (K) extracts 2 h after injection with TNF (+) or with phosphate-buffered saline (−). Results shown are representative of two sets of experiments. Competition assays (lanes 8 to 10) were performed by incubating extracts with excess unlabeled oligonucleotides containing mutant (lane 8) or wild-type NF-κB binding sites (lane 9) or no competitor (lane 10) with kidney extracts from mice injected with TNF, wt, wild type; mut, mutant.

**FIG. 6.** Effect of TNF and transplantation on HCMV ie1 gene expression in vivo. (A) Histochemical analysis of expression of the β-galactosidase transgene under the control of the HCMV major ie promoter in the kidneys of control (left) and TNF-injected (right) mice. (B) Quantitative chemiluminescence analysis of β-galactosidase activity in kidneys of control mice and mice injected with TNF, in kidneys transplanted into allogeneic recipients, and in contralateral control kidneys. Values shown are average light units per nanogram of protein plus standard deviation. TNF (P = 0.01, two-tailed t test) and allogeneic transplantation (P = 0.001, paired t test) induce a statistically significant increase in β-galactosidase activity over that of controls. allo tx, allogeneic transplant.
show any induction of the transgene and were not pursued further.

In order to investigate the response of the HCMV ie promoter/enhancer to allogeneic stimulation, kidneys from transgenic mice were transplanted into allogeneic C57/HeSnJ (H-2^k) recipients, and the level of ß-galactosidase activity was compared with that found in the control contralateral kidney. Because the transgenic mice were derived by injecting the transgene into the ova of mice heterozygous at the H2 locus, it was not possible to perform syngeneic transplants with these mice. However, it is clear from a comparison of the transplanted and contralateral control kidneys that allogeneic transplantation resulted in a statistically significant induction of ie promoter/enhancer activity of approximately threefold at 2 days post-transplantation (P = 0.001). While differences in the context of a gene may influence its expression, the results with the MIEP-lacZ transgenic mice indicate that both TNF and allogeneic transplantation induce HCMV ie gene expression. These results are consistent with our studies of latently MCMV-infected mice, in which the ie promoter/enhancer is in its natural context in the viral genome, and with previous studies of regulation of the HCMV ie promoter/enhancer (54, 67).

**DISCUSSION**

Reactivation of CMV from latency is a frequent complication of organ transplantation, resulting in significant morbidity and mortality. The molecular mechanism by which this occurs is largely unknown. Because the ie1 gene either is not expressed or is expressed at very low levels in latently infected cells (5, 34, 37, 49, 63, 71) and ie1 expression is required to initiate viral replication, induction of ie1 gene expression may be a crucial first step in the reactivation process. In this report we have demonstrated that allogeneic transplantation of kidneys induces expression of the MCMV ie1 gene. This induction occurs only in allogeneic and not syngeneic transplants, indicating that the induction is due to the allogeneic response rather than to ischemia/reperfusion injury. Some IE1 RNA expression was observed in some of the latently infected controls. CMV is known to reactivate periodically, and the observed expression of IE1 RNA may reflect the foci of nascent reactivation observed by others (37). Regardless of whether IE1 RNA was expressed in latently infected mice, it is clear from comparison of RNA expression in the transplanted and contralateral control kidneys that allogeneic transplantation induces MCMV ie1 gene expression. Using transgenic mice harboring the ß-galactosidase gene under the control of the HCMV ie promoter/enhancer, we find that allogeneic transplantation also induces HCMV ie gene expression.

Expression of the MCMV and HCMV ie genes is controlled by the ie promoter/enhancer region (8, 20, 68, 72). The HCMV ie promoter/enhancer consists of repeats of 18-, 19-, 16-, and 21-bp motifs. The 18-, 19-, and 21-bp elements contain binding sites for NF-κB, ATF(CREB), and Sp1, respectively, and the NF-κB and ATF(CREB) sites have been demonstrated to play an important role in regulation of ie gene expression (32, 41, 60, 68). The transcription factors binding to sites in the MCMV ie promoter/enhancer and the sites important in regulating MCMV ie gene expression have not been studied. The MCMV promoter/enhancer (20) has five sites matching the AP-1 consensus site TGANT(C/A)A (39, 40), four AP-1 sites juxtaposed with classic NF-κB sites (GGGRNNYYCC) (2) and an AP-1 site paired with an inverted NF-κB site. While the HCMV promoter/enhancer (8, 72) has only one AP-1 site, it has paired NF-κB/ATF(CREB) sites and single ATF(CREB) sites matching the consensus sequence TGACGTCA (41). AP-1 and ATF (CREB) are a family of related transcription factors which share subunits and bind to similar sequences (28, 39, 43). These observations suggest that although the configuration of the transcription factor binding sites is somewhat different, regulation of the HCMV and MCMV ie genes may be similar.

NF-κB is a family of dimeric transcription factor complexes consisting of p50, p52, p65 (RelA), c-Rel, and RelB subunits. In normal tissues, NF-κB is inactive (6, 22, 44, 69, 81) because it is sequestered in the cytoplasm by the inhibitory subunit IκB (reviewed in references 2 and 3). NF-κB is activated by exposure to lipopolysaccharide or inflammatory cytokines such as TNF or IL-1, viral infection, B- or T-cell activation, and oxidative stress. These diverse stimuli activate different signaling pathways which result in phosphorylation and degradation of IκB, allowing free NF-κB to translocate to the nucleus and activate transcription of NF-κB-responsive genes. These include many of the genes involved in mediating immune and inflammatory responses, including proinflammatory cytokines, chemokines, adhesion molecules, inflammatory enzymes, and receptors (reviewed in reference 3).

During productive HCMV infection, contact between the viral glycoproteins gB and gH and receptors on the cell surface induces activation of NF-κB (79). Activated NF-κB and pp71, the viral transactivator protein released from the virion, translocate to the nucleus and activate the major ie promoter (reviewed in reference 50). Viral components which activate NF-κB during productive infection are not present in latently infected cells. Our model (Fig. 7) postulates that in a transplant recipient, cellular factors activated as a result of allogeneic stimulation and ischemia/reperfusion injury substitute for these viral components in activating the transcription factors required for ie gene expression.

Allogeneic transplantation induces expression of a complex array of genes involved in an inflammatory immune response, including cytokines which activate transcription factors which in turn induce expression of additional genes (10, 14, 15, 24, 56, 69, 81). Previous investigators have noted an association be-
between TNF levels and reactivation of CMV in transplant recipients and suggested that TNF may play an important role in reactivation by inducing ie gene expression through activation of NF-κB (19, 23, 54, 55, 67). We have demonstrated that allogeneic transplantation induces expression of TNF and ie1 and activation of NF-κB in the kidney and that the kinetics of induction are consistent with the hypothesis that TNF mediates induction of ie gene expression through activation of NF-κB in vivo. In contrast, other cytokines which are induced by allogeneic transplantation, such as IFN-γ and IL-2, activate other signaling pathways and are unlikely to play a role in inducing CMV ie gene expression. IL-1, which can induce activation of NF-κB, is not significantly induced by allogeneic transplantation. Preliminary investigation of the effects of IL-1 and IFN-γ on MIEP-lacZ transgene expression showed no induction (data not shown). Thus, our model postulates that TNF plays an important role in mediating induction of ie gene expression in an allogeneic transplant.

We have tested the ability of TNF to induce ie gene expression in vivo by injecting latently MCMV-infected mice and MIEP-lacZ transgenic mice with TNF. We find that TNF alone is able to induce expression of the MCMV ie1 gene in the lungs of latently infected mice, although it appears to be insufficient to induce expression of IE1 RNA in other tissues. Gel shift analysis of the transcription factors activated by allogeneic transplantation and by TNF revealed that allogeneic kidney transplantation activated AP-1 as well as NF-κB. Both of these transcription factors were also activated by TNF in the lung, but only NFκB was activated by TNF in the kidney. The MCMV ie promoter/enhancer has multiple repeats of AP-1 and NFκB binding sites separated by 3 nucleotides. This suggests that AP-1 and NFκB could act cooperatively to control ie gene expression. Synergistic interactions between NFκB and AP-1 have been previously demonstrated (45, 66). Taken together, our results suggest that activation of both AP-1 and NFκB is required for induction of the MCMV ie promoter/enhancer and that activation of AP-1 in the kidney occurs at least in part as a result of ischemia/reperfusion injury and is not mediated by TNF. In contrast, TNF alone was sufficient to induce expression of the HCMV ie promoter/enhancer in the kidneys of MIEP-lacZ transgenic mice. These results are consistent with previous reports that TNF induces expression of the HCMV ie gene expression in vitro (54, 67). Gel shift analysis of extracts from MIEP-lacZ transgenic mice injected with TNF demonstrated that as is the case with BALB/c mice latently infected with MCMV, TNF induces activation of both NFκB and AP-1 in the lung but of NFκB alone in the kidney (data not shown). The differences in the response of the transgene and the MCMV ie1 gene to TNF in the kidney are therefore likely to reflect differences in the promoters of these genes rather than differences in the strains of mice in TNF-mediated signal transduction.

Thus, our results are consistent with a model for reactivation from latency in which TNF released as a result of allostimulation activates the transcription factors which drive ie gene expression, which in turn activates lytic replication of the virus (Fig. 7). These studies will provide the framework for future studies using genetically deficient strains of mice to further define the components required for this induction.

Although we observed induction of ie1 gene expression in transplants of mice latently infected with MCMV, expression of IE3, an alternatively spliced transcript initiated at the ie1 promoter, was not observed. This may be due to differences in sensitivity of detection. Alternatively, some investigators have suggested that posttranscriptional regulation of ie3 (or ie2 in the case of HCMV) may play an important role in inducing reactivation (38, 71). Expression of genes representative of later phases of MCMV replication, E-1 and gB, was also not observed. This is likely due to immune destruction of cells expressing foreign antigens. Complete reactivation of HCMV with production of infectious virus can be achieved in vitro by allogeneic stimulation of latently infected peripheral blood mononuclear cells (64). Reactivation of infectious virus has also been demonstrated in animal models by allogeneic transplantation or cell transfer combined with immunosuppression (11, 12, 47, 61, 76). Thus, it is likely that reactivation is a multistep process which is initiated by induction of ie gene expression and that immunosuppression is required to achieve full lytic replication in vivo. Further studies will be required to determine the roles of the immune response and additional factors in achieving complete reactivation in vivo.

The ability to establish a lifelong latent infection and to reactivate periodically is a characteristic common to all herpesviruses. Presumably, this ability has evolved because it confers a selective advantage to the virus (58). TNF is released by activated T cells and macrophages as part of the inflammatory response to infection. Reactivation of viral replication in response to inflammation would allow the virus to escape from a host which might succumb to other infections and thus would be expected to have an important survival advantage. Furthermore, a host suffering from acute infection is likely to be immunocompromised, and reactivation under these circumstances would favor virus escape over immune destruction. The observation that reactivation of CMV is associated with sepsis (19) as well as with allogeneic transplantation supports the hypothesis that the natural stimulus for reactivation is an inflammatory immune response and that allogeneic transplantation mimics this process. The observations that reactivation of Epstein-Barr virus occurs as a result of B-cell activation (which would occur during infection) and that infection or tissue damage (75) induces reactivation of herpes simplex virus (hence the terms “cold sore” and “fever blister”) suggest that reactivation in response to inflammatory mediators may be a more general strategy which has contributed to the success of herpesviruses as pathogens.

ACKNOWLEDGMENTS

This work is supported by NIH grant R01AI42898–02 to M.I.A. We thank the members of the Transplant Division for advice and support, Jay Nelson for MIEP-lacZ transgenic mice, Dave Ivancie for help with histology, Laimonis Laimins for luminometer use, Sarah Martin for early analysis of transgenic mice, Patricia Spear and Richard Lengnecker for advice and critical review of the manuscript, and Wei Hsueh for support and use of laboratory space.

REFERENCES