

Chemokine Sequestration by Viral Chemoreceptors as a Novel Viral Escape Strategy: Withdrawal of Chemokines from the Environment of Cytomegalovirus-infected Cells

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Summary

Human cytomegalovirus (HCMV), a betaherpesvirus, has developed several ways to evade the immune system, notably downregulation of cell surface expression of major histocompatibility complex class I heavy chains. Here we report that HCMV has devised another means to compromise immune surveillance mechanisms. Extracellular accumulation of both constitutively produced monocyte chemoattractant protein (MCP)-1 and tumor necrosis factor–superinduced RANTES (regulated on activation, normal T cell expressed and secreted) was downregulated in HCMV-infected fibroblasts in the absence of transcriptional repression or the expression of polyadenylated RNA for the cellular chemokine receptors CCR-1, CCR-3, and CCR-5. Competitive binding experiments demonstrated that HCMV-infected cells bind RANTES, MCP-1, macrophage inflammatory protein (MIP)-1 β , and MCP-3, but not MCP-2, to the same receptor as does MIP-1 α , which is not expressed in uninfected cells. HCMV encodes three proteins with homology to CC chemokine receptors: US27, US28, and UL33. Cells infected with HCMV mutants deleted of US28, or both US27 and US28 genes, failed to downregulate extracellular accumulation of either RANTES or MCP-1. In contrast, cells infected with a mutant deleted of US27 continues to bind and downregulate those chemokines. Depletion of chemokines from the culture medium was at least partially due to continuous internalization of extracellular chemokine, since exogenously added, biotinylated RANTES accumulated in HCMV-infected cells. Thus, HCMV can modify the chemokine environment of infected cells through intense sequestering of CC chemokines, mediated principally by expression of the US28-encoded chemokine receptor.

Key words: RANTES • human cytomegalovirus • chemokine receptors • sequestration • monocyte chemoattractant protein 1

Efficient control of persistent viral infections relies on the specific recognition of viral antigens by T lymphocytes and/or NK cells. These cells are activated by inflammatory cytokines. However, once activated they must be mobilized to the sites of infection. Such mobilization depends on the creation of gradients of chemokines that not only chemoattract effector leukocytes, but also play a supplementary role in stimulating the effector mechanisms of these cells once they are targeted to sites of infection (1).

Numerous viruses persist in host organisms for the remainder of the individual's life. This persistence requires

the virus to be able to evade eradication by the immune system and reflect a co-evolution of the infectious agent with its host. A number of viruses have developed a variety of mechanisms for immune evasion (2–5). Some of these viruses confer lifelong immunity, as assessed by persistent immunological markers of primary infection, without complete eradication of the virus in question. To accomplish this feat, they have acquired genes that mimic host genes involved in the immune response (for review see references in 5, 6) and have products that can interfere with numerous host cell defense mechanisms (complement activation, in-

terferon inhibition, antibody recognition, growth factors and cytokines, etc.)

Notable among such persistent viruses are the herpesviruses (2, 4, 7). After primary infection, these viruses become permanent residents of their hosts. In the majority of cases, primary infection is clinically inapparent. Pathophysiological manifestations are only apparent upon immunodepression of the host, whether natural (pregnancy), iatrogenic (organ transplantation), or acquired (infection with HIV). From a purely virological point of view, this implies that these viruses have developed a low profile as concerns the immune system.

Herpesviruses are among the largest viruses known. Their genomes have the capacity to encode from 80 to >200 proteins (7). They have evolved with their hosts for millions of years (8). The target cells of viral latency are sometimes very limited, for instance for herpes simplex virus (neurons) and Epstein-Barr virus (B lymphocytes). Latency targets of viruses like CMV (9–11), appear much broader. In this case, the virus has probably adopted multiple strategies for evading immune surveillance, since it resides in a variety of functionally different cell types.

Human CMV (HCMV),¹ a betaherpesvirus, has developed a number of strategies which may limit its recognition by the host's immune system and its eradication. It induces expression of Fc receptors (12–15) and upregulates expression of complement regulatory proteins CD55 and CD46 (16), and incorporates CD55 and CD59 into the virion envelope (17). The HCMV genome also contains a number of genes that code for factors capable of interfering with immune surveillance. Most renowned are the four genes (US2, US3, US6, and US11) that code for proteins that prevent presentation of HLA class I molecules at surface of infected cells (18–23). HCMV also encodes its own HLA class I-like molecule that recently has been shown to act as a decoy for NK cells (24, 25). Finally, HCMV virus potentially encodes a protein homologous to the variable and constant regions of the TCR- γ chain (UL20) (26), the function of which has yet to be determined.

In addition to the aforementioned genes, HCMV possesses three genes, designated UL33, US27, and US28, that encode proteins homologous to the seven transmembrane domain CC chemokine receptors (27, 28). To date, only one of these receptors, US28, has been shown to be a functional CC chemokine receptor (29, 30).

In this study, we explored the functionality of US28 and US27 in the context of HCMV-infected cells and we show that constitutively produced or cytokine-superinduced CC chemokines are depleted from infected cell medium. Our data suggest that the HCMV-encoded US28, and perhaps

US27, G protein-coupled receptor homologues have a role in this process. The results indicate that HCMV has devised another strategy that may play a role in immune system evasion by reducing the availability of host chemokines.

Materials and Methods

DNA Sequence. The nucleotide numbering system of Chee et al. (28) was used for the HCMV strain AD169 DNA sequence (available from EMBL/GenBank/DBJ under accession number X17403).

Cells. Human foreskin fibroblast (HFF) cells and human diploid lung fibroblast (MRC-5 and WI-38) cells were used in this study. All cells were grown in DMEM containing 5–10% FCS. Cells were consistently negative for mycoplasma.

Virus. The AD169 strain of HCMV was used throughout as wild-type HCMV and as the parent strain for the development of the recombinant mutant viruses described below. A low passage HCMV clinical strain and simian CMV were provided by J. Nelson (Oregon Health Sciences University, Portland, Oregon) and W. Gibson (Johns Hopkins University School of Medicine, Baltimore, MD), respectively. Viral growth, titrating, and the production of stocks in fibroblasts have been described previously (31). Mutant viruses were derived by insertion of a 2.85-kb β -glucuronidase (β -gluc) expression cassette (β -gluc is under the control of the early HCMV 2.7-kb promoter and the herpes simplex virus type 1 thymidine kinase polyadenylation signal) into cloned viral target genes and subsequent homologous recombination into the viral genome, using a strategy described previously (18, 20, 32). Control mutant virus RV134 contains the β -gluc cassette insert within the US9-US10 intergenic region at base 199,021 (32), such that no genes or transcription units were interrupted (33). The β -gluc cassette insertion in RV91 is at base 218,251, disrupting the 1.09-kb US27 gene \sim 0.35 kb from its translation initiation codon. RV92 contains the β -gluc cassette in place of a 0.20-kb NH₂-terminal region (bases 219,426–219,629), 0.23 kb from the translation initiation codon of the 1.06-kb US28 gene. The β -gluc cassette insertion in RV101 replaces bases 218,251–219,629, thereby deleting the 0.74- and 0.43-kb regions of US27 and US28, respectively. Plaques containing β -gluc-expressing virus were purified and the proper genomic organization of each mutant was determined by DNA blot hybridization (32) to reveal diagnostic BamHI and EcoRI restriction fragment changes. Viral infections for single cycle growth analyses in HFFs were done at a multiplicity of infection of 2; total virus yield was assessed from day 2 to day 7 postinfection (pi), as described previously (32).

Measurement of Chemokines. RANTES (regulated on activation, normal T cell expressed and secreted) and monocyte chemoattractant protein (MCP)-1 were measured by ELISA using kits (purchased from Medg enix-Biosources [Rungis, France] or R&D Biotechnology [Abingdon, UK] for RANTES, and Hy-Cult Biotechnology [Uden, The Netherlands] and R&D Biotechnology for MCP-1) according to the manufacturers' instructions. Intracellular RANTES was measured in cytosolic fractions after treatment of cells with acid-glycine (34) buffer for 3 min at room temperature, trypsinization, and lysis in 10 mM hepes, pH 8, 1 mM EDTA, 50 mM NaCl, 0.5 M sucrose, 0.5% NP-40, 1 mM PMSF, and 1% digitonin. All results are expressed as picogram of chemokine per milliliter.

Chemokines and Specific Antibodies. Anti-human RANTES and anti-human MCP-1 were purchased from R&D Biotechnology. For infected cell binding studies, ¹²⁵I-labeled MIP-1 α (2,200Ci/

¹Abbreviations used in this paper: β -gluc, β -glucuronidase; Fmoc, fluorenylmethylloxycarbonyl; HCMV, human cytomegalovirus; HFF, human foreskin fibroblast; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; pi, postinfection; RANTES, regulated on activation, normal T cell expressed and secreted; RANTES-B, biotinylated recombinant human RANTES; RT-PCR, reverse transcriptase PCR.

mmol) was purchased from NEN™ Life Science Products (Boston, MA); unlabeled chemokines MCP-1, MCP-2, MCP-3, RANTES, MIP-1 α , and MIP-1 β were purchased from Pepro Tech (Rocky Hills, NJ).

Biotinylation of RANTES. RANTES was obtained by solid phase synthesis, using the fluorenylmethyloxycarbonyl (Fmoc)-chemistry on a fully automated Synthesizer (Applied Biosystems model 433A; PE Applied Biosystems, Foster City, CA), and biotin was specifically incorporated after completion of the synthesis, according to the protocol described previously (35). Purity and identity of the product were assessed by analytical HPLC, capillary electrophoresis, amino acid analysis, and electrospray mass spectrometry.

Chemokine Binding Experiments. All assays were done in 24-well tissue culture plates containing $\sim 1.5 \times 10^5$ HFF cells per well as a confluent monolayer. Growth media was removed from uninfected or virus-infected (multiplicity of infection 2.5) HFF cells at the desired time. Each well was washed two times with cold (4°C) PBS. 0.25 ml of cold binding buffer (DMEM containing 25 mM Hepes and 1 mg/ml BSA) was added to each well. Where indicated, cold competitor chemokine was added (4 μ l per well) and the plate was incubated at 4°C for 15 min with gentle rocking. Then, 2.5 μ l of 125 I-labeled MIP-1 α (0.25 nM final concentration) was added to each well and the plate was incubated at 4°C for 6 h. The media was removed and the monolayer was washed twice with cold binding buffer, then once with cold PBS containing 1 mg/ml BSA. 1 ml of extraction buffer (20 mM sodium phosphate, pH 7.5, with 1% Triton X-100) was added to each well and incubated at 37°C for 30 min. The extracts were then counted in a gamma radiation counter (model 1272; LBK; Valac, Gaithersburg, MD).

Immunoblot Analysis. HCMV UL80 protease expression was assessed by ECL (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) immunoblot analysis using UL80 16K rabbit polyclonal primary antibody, as described previously (36).

Induction of RANTES Production by TNF Treatment of Fibroblasts. Fibroblasts were seeded at 10^5 cells/well of 24-well plates. The next day, some cells were infected with HCMV (multiplicity of infection 5). Where indicated, uninfected or infected cells were treated with 5 ng/ml TNF- α . TNF- α treatment of uninfected and infected cells was continuous for 24, 48, or 72 h. Culture supernatants were collected at 0–24, 24–48, and 48–72 h after infection and/or the beginning of treatment, clarified, and frozen at -20°C before measuring RANTES. Cells were re-fed with fresh medium, either with or without TNF- α , depending on the length of treatment, as indicated in the legend of Fig. 1.

Adsorption of RANTES by Infected Cells. Before adsorption, supernatants were collected and cells were washed once with PBS and then incubated for 3 min at room temperature in acid-glycine buffer (34) to remove residual receptor-bound chemokines. Cells were washed once again with PBS before incubation with RANTES. Recombinant human RANTES (rRANTES) at 500 pg/ml was adsorbed to uninfected cells or cells infected for 24, 48, or 72 h after infection at 37°C for time intervals indicated in Results. Cell medium was then sampled, clarified by centrifugation at 5,000 rpm for 5 min, and maintained at -20°C before measurement of RANTES. The starting RANTES solution was also frozen.

For internalization of biotinylated-rRANTES (RANTES-B), 48 h after infection with recombinant and wild-type HCMV, cells were washed and treated with acid-glycine buffer as above, and then incubated with 100 nM RANTES-B for 3 h at 37°C. Cells were washed once with PBS, incubated 5 min with glycine-

buffer to remove membrane-bound biotinylated-rRANTES, and then lysed in high salt buffer (50 mM Tris, pH 8, 300 mM NaCl, 10% (vol/vol) glycerol, 0.5% NP-40, 1% digitonin, and 1 mM PMSF). Proteins were separated in 15% SDS-PAGE and transferred to reinforced nitrocellulose (Sartorius, Göttingen, Germany). Blots were saturated with 3% cold-water fish gelatin, 1% BSA (both from Sigma Chemical Co., St. Louis, MO) in PBS/Tween 20 (0.1%). Blots were then incubated for 1 h at room temperature with a 1:300 dilution of peroxidase-labeled streptavidin (Amersham Pharmacia Biotech) in the same buffer before being developed by enhanced chemiluminescence (Super ECL; Pierce Chemical Co., Rockford, IL).

RNA Analysis. Expression of transcripts from HCMV UL33, US27, and US28 genes were analyzed by RNA blot hybridization using riboprobes, as described previously (33). Reverse transcriptase (RT)-PCR was used to examine expression of cellular chemokines and chemokine receptors. For RT-PCR, RNA was extracted using the RNeasy™ kit (QIAGEN S.A., Courtaboeuf, France), and polyadenylated mRNA was purified using the Oligotex Direct mRNA kit (QIAGEN S.A.). This RNA was then treated with RNase-free DNase I (Boehringer Mannheim, Meylan, France) for 1 h at 37°C and repurified using the RNeasy™ kit, and the yield was quantified spectrophotometrically. RT-PCR was conducted using 500 ng of RNA as described by Kawasaki (37). As a positive control, human β -actin RNA was retrotranscribed and amplified in parallel for each sample. As a negative control, an identical amount of RNA was amplified without being retrotranscribed. PCR was conducted for 35 cycles according to the protocol described by Michelson et al. (38). Transcription of polyadenylated RNA was performed by RT-PCR for RANTES and MCP-1 at 48 and 72 h after HCMV infection using the following primers: RANTES sense, 5' CGG GAT CCA TGA AGG TCT CCG CGG CA 3'; RANTES antisense, 5' CGG AAT TCC TAG CTC ATC TCC AAA GA 3'; MCP-1 sense, 5' GCC GCC CTT CTG TGC CTG CTG CTC ATA GCA 3'; and MCP-1 antisense, 5' GGG GTA GAA CTG TGG TTC AAG AGG AAA AG 3'. Transcription analysis of cellular CC chemokine receptors CCR1, CCR3, and CCR5 and polyA RNA for CCR1 was performed using whole cell RNA extracted and from uninfected cells and cells infected with HCMV for either 4 or 24 h. Primers for these receptors were as follows: CCR-1 sense, 5' AAGATTCTGCTAAGACGACC 3'; antisense, 5' ATCACCTCCGTCACCTTGC 3'; CCR-3 sense, 5' GAATGACCATCTTCTGTCTCG 3'; antisense, 5' GAAGGATAGCCACATTGTAGG 3'; CCR-5 sense, 5' CTGACATCTACCTGCTCAACC 3'; and antisense, 5' GAAGATTCCAGAGAA-GAAGCC 3'.

Results

Depletion of TNF-induced RANTES from Medium of HCMV-infected Fibroblasts. HCMV infection of HFF cells induces RANTES production, but as the virus replication cycle progresses, extracellular RANTES is no longer detected, although RANTES transcription continues (38). To see if cytokine-superinduced RANTES production is similarly depleted from HCMV-infected cell medium, uninfected and infected HFF cells were treated continuously with TNF- α for 24, 48, or 72 h and culture medium was collected at the indicated intervals (Fig. 1). At each time interval, cells were washed and re-fed medium with or with-

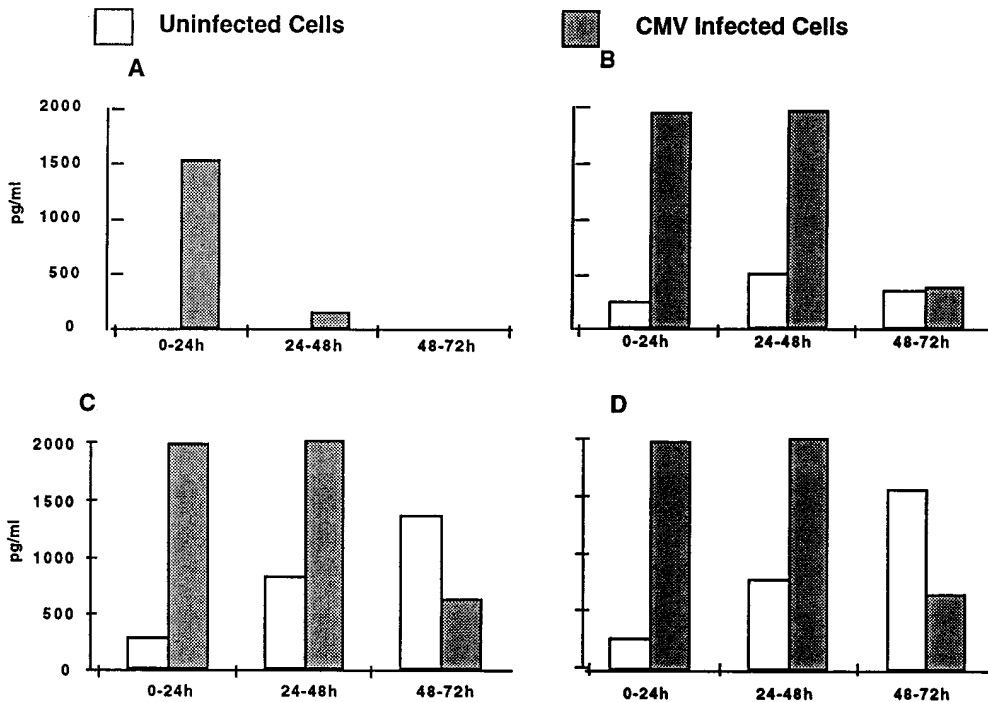


Figure 1. Uninfected and infected cells were incubated with TNF (5 ng/ml) from time 0 (relative to the start of infection) for varying periods of time: A, no treatment with TNF; B, treated with TNF from 0 to 24 h; C, treated with TNF from 0 to 48 h; D, treated with TNF from 0 to 72 h. RANTES production was measured in culture supernatants collected from infected and uninfected cells at the intervals indicated in the x axis (0–24, 24–48, and 48–72 h relative to the start of infection or the beginning of treatment). At each time, cells were washed and re-fed medium with or without TNF as indicated. The experiment was run in triplicate. Extracellular RANTES (pg/ml) was quantified by ELISA.

out TNF (5 ng/ml), as indicated in the legend to Fig. 1. Uninfected, nontreated cells produced no detectable extracellular RANTES throughout the observation period. In contrast, RANTES production was induced by HCMV infection at 24 h pi, but decreased to undetectable levels by 72 h pi (Fig. 1 A), in accordance with our earlier findings (38). Medium from uninfected cells treated for 24 h with TNF- α contained ~200 pg/ml of RANTES (Fig. 1 B). This production increased slightly after the removal of TNF- α . When HCMV-infected cells were treated with TNF- α for 24 h (starting from the time of virus adsorption to cells), the level of extracellular RANTES accumulation within the 0–24 h period was ~20% higher than that produced by nontreated, infected cells (1,900 pg/ml compared with 1,500 pg/ml). However, as a function of time after infection, extracellular RANTES accumulation decreased markedly in the medium of all infected cell cultures, dropping to below 400 pg/ml. When TNF- α treatment was maintained for 48 or 72 h, RANTES accumulation increased steadily in the medium of uninfected cells (Fig. 1, C and D). From a baseline of 200 pg/ml after 24 h of treatment, RANTES levels increased to 1,300 and 1,500 pg/ml by 72 h when cells were continuously treated for 48 and 72 h, respectively. In contrast, in the medium of similarly treated HCMV-infected cells, extracellular levels of RANTES declined steadily from 1,950 pg/ml to ~650 pg/ml, a threefold decrease. This decrease was not attributable to repression of RANTES transcription, as shown by RT-PCR analysis of polyadenylated RNA from infected cells (Fig. 2).

Depletion of Constitutively Produced MCP-1 from Medium of HCMV-infected Fibroblasts. During the course of our stud-

ies, we observed that our HFF cells produced MCP-1 constitutively (Fig. 2). Therefore, we asked whether HCMV infection could also lead to the depletion of this constitutively produced CC chemokine. Infection of HFF cells with either pelleted AD169 virions or whole AD169 inoculum led to a 10- and 20-fold reduction, respectively, of extracellular MCP-1 by 72 h pi (Table 1). This did not occur when fibroblasts were incubated with virus-free supernatant or infected with heat- or UV-inactivated virus. Two other human diploid fibroblast lines that we examined, MRC-5 and WI-38, also produced MCP-1 constitutively. Infection of both these lung fibroblasts with whole viral inoculum resulted in a 5–10-fold reduction in extracellular MCP-1 levels by 48 h pi (data not shown). Analysis by RT-PCR of polyadenylated RNA extracted from unin-

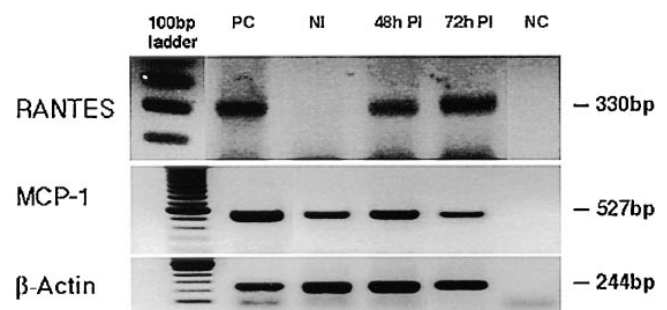


Figure 2. Cells were infected with wild-type HCMV for the times indicated (PI) or were uninfected (NI). Poly A RNA was extracted, reverse transcribed, and amplified by PCR using primers specific for RANTES, MCP-1, or actin. PC, positive control of the corresponding cDNA cloned in pCDNA; NC, the negative control consisting of nonretrotranscribed poly A RNA. Molecular weight markers were the 100-bp ladder.

Table 1. Extracellular Production of MCP-1 after HCMV Infection of Fibroblasts

| Fibroblasts | Extracellular production of MCP-1 | | | |
|------------------------|-----------------------------------|---------------------|----------------------|----------------------|
| | 0–8 h [‡] | 8–24 h [‡] | 24–48 h [‡] | 48–72 h [‡] |
| | <i>pg/ml</i> | | | |
| Uninfected | 5,615 | 11,840 | 9,938 | 10,879 |
| Virus-free supernatant | 10,016 | 10,738 | 10,972 | 12,131 |
| Infected with: | | | | |
| UV-inactivated | 9,906 | 10,572 | 10,889 | 10,555 |
| Heat-inactivated | 10,946 | 10,551 | 10,136 | 12,021 |
| Pelleted virus | 8,992 | 9,377 | 5,329 | 1,157 |
| Whole inoculum | 10,811 | 10,000 | 4,301 | 570 |

*Representative of two experiments.

[‡]Time intervals at which supernatants were collected. At each time interval, cells were washed and re-fed growth medium.

ected and HCMV-infected HFF cells demonstrated that depletion of MCP-1 from culture medium was not due to cessation of transcription (Fig. 2).

Measurement of Intracellular Levels of RANTES and MCP-1. To determine if these chemokines could be detected intracellularly, supernatants of uninfected and infected cells were collected. The corresponding cells were incubated in acid-glycine buffer to remove receptor-bound chemokine followed by extraction of a cytoplasmic fraction (see Materials and Methods). RANTES and MCP-1 were measured in duplicate supernatant/cytoplasm pairs by ELISA. At 24 h pi (Table 2), when extracellular chemokine levels are highest, intracellular chemokine represented 32 and 17% of extracellular RANTES and MCP-1 levels, respectively. At 48 h pi, the levels of intracellular chemokines were below the level of detection by ELISA.

Chemokine Binding to HCMV-infected Cells. The HCMV genome encodes three proteins with homology to CC type chemokine receptors: UL33, US27, and US28 (39). To determine when these genes may function during the course of the viral replicative cycle, their expression in HCMV strain AD169-infected HFF cells was examined by RNA blot hybridization (Fig. 3). A previous study indicated that each of these genes was expressed by late times pi (40). Our analyses revealed that both UL33 (3.0–3.3 kb) and US28 (1.3 kb) transcripts are detected much earlier, by 8 h pi, and increase in abundance to 72 h pi (i.e., early and late times pi, respectively). In contrast, US27 (2.9 kb) RNA was detected only at late times pi (i.e., 48–72 h pi). US27 RNA is detected with the same probe used for US28, since the transcript that initiates just upstream of US27 reads through both US27 and US28 (40) (Fig. 3 B).

Of the three HCMV-encoded G protein-coupled CC chemokine receptor homologues, only the US28 gene product has been shown to be a functional chemokine receptor, and only in transfected cells (29, 30). These reports

Table 2. Measurement of Intracellular RANTES and MCP-1

| Infection | RANTES | | MCP-1 | |
|-----------|---------------|---------------|---------------|---------------|
| | Extracellular | Intracellular | Extracellular | Intracellular |
| | <i>pg/ml</i> | | <i>pg/ml</i> | |
| none | 0 | 0 | 2,306 | 94 |
| AD169 | 994 | 320 | 2,423 | 418 |

At 24 h after infection, culture supernatants were collected and cells were treated with glycine buffer to strip cell membranes of chemokines before lysing. Concentrations of chemokine are expressed as picogram/milliliter. All points represent the average of duplicate supernatant/cytoplasm pairs.

indicated that, at least in the absence of other HCMV gene products, the US28-encoded receptor could bind the CC chemokines MIP-1 α , MIP-1 β , MCP-1, and RANTES. We sought to extend these observations by examining the binding of the CC chemokine, MIP-1 α , to HCMV strain AD169-infected HFF cells at 24, 48, and 72 h pi. (Fig. 4 A). Uninfected fibroblasts did not bind MIP-1 α , whereas binding to HCMV-infected cells was detected at all three times, increasing from early (24 h) to late times (72 h) pi. Indicative of specificity, this binding was reduced to background levels (i.e., 10–40-fold reduction) in the presence of 400-fold excess unlabeled MIP-1 α , similar to those levels detected in uninfected cells. Thus, in both qualitative and quantitative terms, the early-late binding kinetics of chemokines to HCMV-infected cells coincides with the early-late expression kinetics of the US28 transcription unit (Fig. 3 B). Similar chemokine binding experiments were performed using a recent clinical isolate of HCMV and simian CMV. Specific MIP-1 α binding to fibroblasts infected with these CMVs was also observed (data not shown). Thus, CC chemokine binding in cells infected with at least primate CMVs is conserved and may be functionally important.

To examine the breadth of CC chemokine binding in HCMV-infected cells to the MIP-1 α receptor, competition binding studies were performed in the presence of 800-fold excess unlabeled heterologous chemokines (Fig. 4 B). This experiment indicated that the CC chemokines MIP-1 β , RANTES, MCP-1, and MCP-3 caused the displacement of >95% of MIP-1 α binding, and thus bind to the same receptor as does MIP-1 α . In contrast, MCP-2 did not compete with MIP-1 α , as indicated by <5% displacement. Furthermore, additional binding experiments indicated that competition for the MIP-1 α receptor in HCMV-infected HFF cells by heterologous CC chemokines occurred in a dose-dependent fashion as expected for receptor-ligand interactions (data not shown). The relative affinity of each chemokine for the MIP-1 α receptor was estimated based on the concentration of cold competitor required to displace 50% of bound ¹²⁵I-labeled MIP-1 α . In these

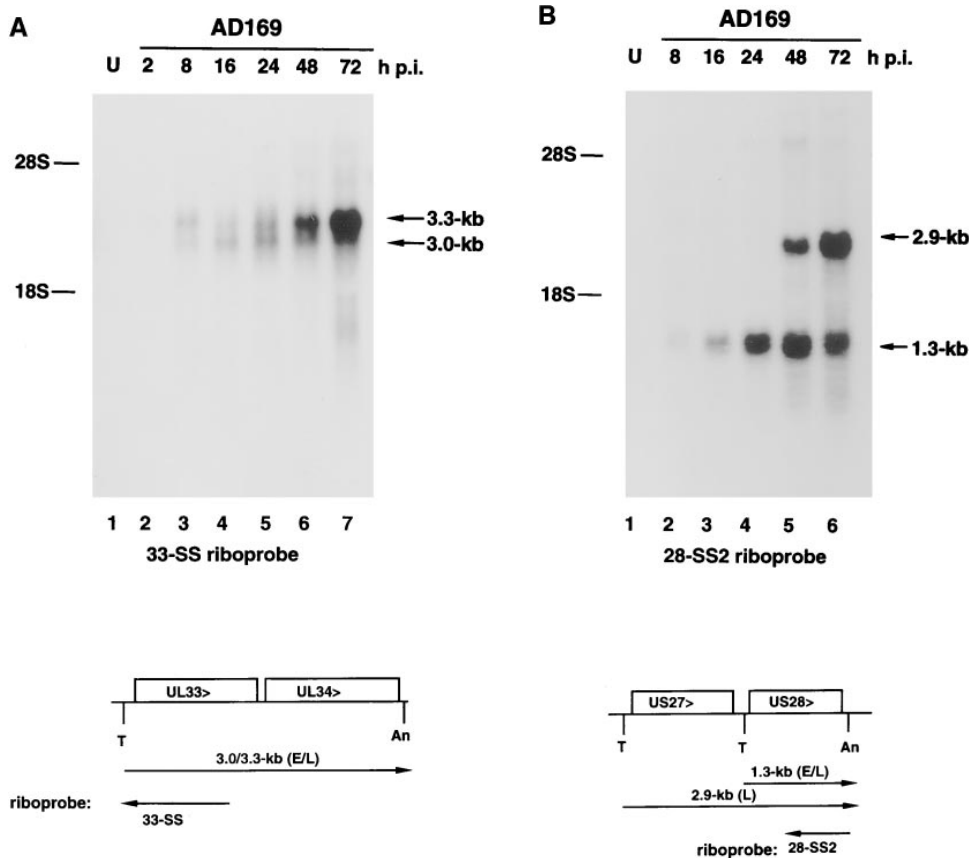


Figure 3. Kinetics of HCMV UL33 (A) and US27/US28 RNA (B) expression. Total cytoplasmic RNA from uninfected (*U*) or HCMV strain AD169-infected HFF cells (multiplicity of infection 3) were isolated at the indicated hour pi and analyzed by blot hybridization. Riboprobes used for each blot, as well as the expected transcripts (40) are indicated in the schematic drawing. *T*, transcription initiation site; *An*, polyadenylation site; *E*, early kinetic class transcript; *L*, late kinetic class transcript.

experiments, unlabeled RANTES, MIP-1 α , and MIP-1 β competed for binding to the MIP-1 α receptor with approximately equal efficiency (~ 1 nM); MCP-1 and MCP-3 competed less well, requiring approximately fivefold higher concentrations.

Construction and Characterization of Mutant HCMV Deleted of Genes Encoding CC Chemokine Receptors. It was of interest to determine whether US28, its related adjacent gene (US27), or UL33 were responsible for either (a) the depletion of CC chemokines from HCMV-infected cell supernatants; or (b) the specific binding of CC chemokines to HCMV-infected cells. For this purpose, insertion or deletion mutants of the HCMV strain AD169 were constructed by insertion of the 2.85-kb β -gluc expression cassette in the US27 or US28 gene loci, as shown (Fig. 5, A–C). RV91 has a disruption of the US27 gene; RV92 has disruption/deletion of US28; and RV101 is deleted of both US27 and US28 (Fig. 5 B). The proper genomic organization of these mutants was verified by both DNA and RNA blot hybridization (data not shown). RV134 has an insertion within the US9–US10 intergenic region and serves as a pseudo wild-type control mutant (32). Single cycle growth analyses (Fig. 5 D) indicated that all viruses grew with the same kinetics, but RV92 had an ~ 5 –10-fold lower yield. The slight yield impairment of RV92 is not due to the deletion of US28, since RV101 (deleted of both US27 and US28) grows to levels similar to AD169 wild-type and

control RV134 viruses, but may be due to a positional effect of the marker gene insertion.

To determine if any of the disrupted or deleted genes in these mutants were responsible for binding chemokines, studies were performed in cells infected with mutant HCMV at 72 h pi (Fig. 6), when chemokine binding to wild-type infected cells is maximal (Fig. 4). HFF cells infected with either of two independent isolates of US28 deletion mutant RV92, designated RV92-2 and RV92-15, failed to bind MIP-1 α (Fig. 6 A). Binding of radiolabeled MIP-1 α to RV92-infected HFF cells was reduced to the background level detected in uninfected cells. In a parallel experiment, immunoblot analyses of the HCMV UL80 protease expression was used to confirm that the late stage of the replicative cycle was reached in cells infected with mutant and wild-type viruses (reference 36 and data not shown). Although the US28 gene is disrupted by the β -gluc expression cassette insertion in RV92, the upstream US27 transcription unit is also altered, since in wild-type HCMV, US27 and US28 transcripts are 3' co-terminal (Fig. 5 B and reference 40). The possible contribution of US27 was addressed in binding experiments using US27 insertion mutant RV91-infected HFF cells (Fig. 6 B). RV91-infected cells bound MIP-1 α to approximately the same extent as did cells infected with either wild-type strain AD169 or control insertion mutant RV134. Furthermore, RV101, a mutant deleted of both US27 and US28,

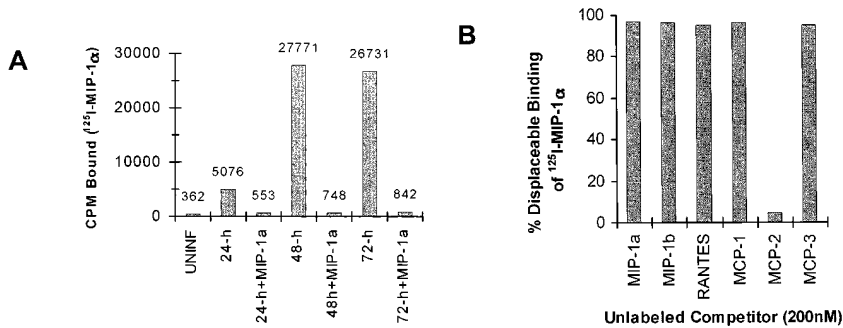


Figure 4. Time course and specificity of MIP-1 α receptor expression. (A) ¹²⁵I-labeled MIP-1 α binding to uninfected (UNINF) or HCMV strain AD169-infected HFF cells was examined at the indicated time pi, in either the absence or presence of excess unlabeled MIP-1 α (*h+MIP-1 α*) at a final concentration of 100 nM. cpm of bound radiolabeled MIP-1 α are given above each bar. (B) Percentage of displaceable binding of ¹²⁵I-labeled MIP-1 α using the indicated unlabeled chemokine (200 nM final concentration) in infected HFF cells at 72 h pi.

also failed to bind MIP-1 α . In contrast, as an additional control, cells infected with RV11, a mutant with a β -gluc insertion replacing an NH₂-terminal portion of UL33 with unimpaired growth kinetics (Jones, T.R., unpublished data), retained full ability to bind MIP-1 α (Fig. 6 B). By RNA blot hybridization, each of the HCMV mutants that bound the CC chemokine MIP-1 α at late times pi had an intact US28 transcription unit which was expressed at wild-type levels (data not shown). Therefore, the data are

indicative that the US28-encoded receptor is in large part responsible for CC chemokine binding in HCMV-infected cells.

Role of Receptors Encoded by US28 in the Depletion of RANTES and MCP-1 after Infection. Three human cellular receptors, CCR1, CCR3, and CCR5, have been shown to bind CC chemokines, including RANTES and MCP-1 (41). However, by RT-PCR, we were unable to detect any transcripts for CCR3 and CCR5 in total RNA or of CCR1 in the polyadenylated fraction from either uninfected or HCMV-infected HFF cells (data not shown). Therefore, the early-late expression kinetics of the HCMV US28-encoded receptor (Fig. 3 B), in conjunction with its capability to bind RANTES and MCP-1 (Figs. 4 and 6), suggest that it may play a role in the depletion of these chemokines from the media of HCMV-infected cells (Table 1 and Fig. 1). To directly study the HCMV encoded CC chemokine receptor homologues in chemokine depletion, the extracellular levels of RANTES and MCP-1 in the culture medium of HFF cells infected with each of the CC chemokine receptor mutants were examined (Fig. 7). Infection with RV92 or RV101, both US28 deletion mutants, failed to lead to the extensive depletion of either RANTES (Fig. 7 A) or MCP-1 (Fig. 7 B) from the culture medium. In contrast, infection with RV91 led to a marked reduction of extracellular RANTES and MCP-1, almost similar to that observed in cells infected with wild-type HCMV. Comparable effects on extracellular RANTES and MCP-1 levels were observed in other fibroblasts, MRC-5 and WI-38, infected with these mutant viruses (data not shown). Consistent with its ability to bind CC chemokines, these results strongly suggest that US28 plays a major role in the depletion of CC chemokines from the extracellular media of HCMV-infected cells.

Internalization of Exogenously Added RANTES by Infected Cells. Previously, we demonstrated by immunofluorescence that RANTES accumulated within HCMV-infected cells concomitant with its depletion from culture medium. Additional experiments indicated that this was not due to soluble proteases (38). These observations raised the following possibilities: (a) RANTES or MCP-1 is being retained intracellularly by HCMV-encoded receptors as these chemokines are synthesized and/or (b) newly synthesized chemokines are released from the infected cell, but that HCMV-encoded cell receptors expressed at the cell surface

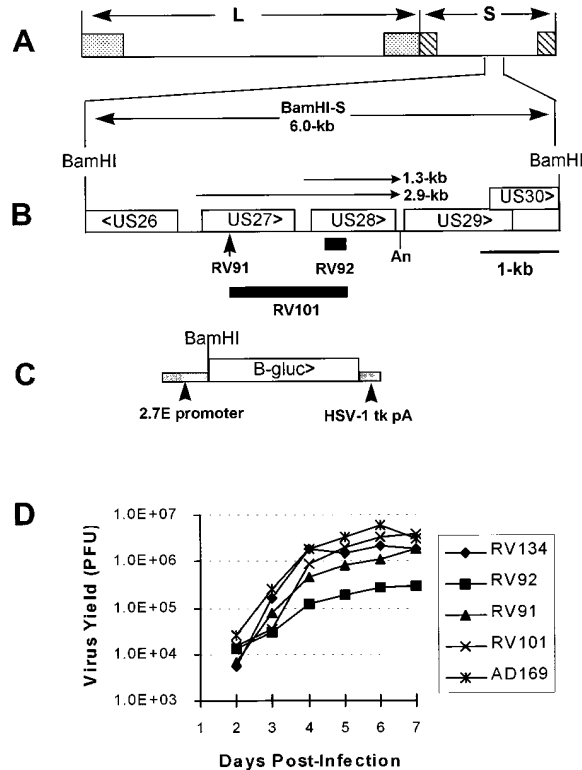


Figure 5. Genome organization and growth of HCMV mutants. The organization of the HCMV genome (A) and the BamHI-S fragment (B), which contains the US27 and US28 genes, are shown in the schematic drawing. In B, the US27 and US28 transcripts are shown, as well as the sites of the β -gluc expression cassette insertions in RV91, RV92, or RV101. The arrowhead is the site of a point insertion (RV91) and dark rectangles indicate deletions (RV92 and RV101). (C) Schematic drawing of the 2.85-kb β -gluc expression cassette used in the construction of HCMV mutants. (D) Single cycle growth analysis of HCMV mutants.

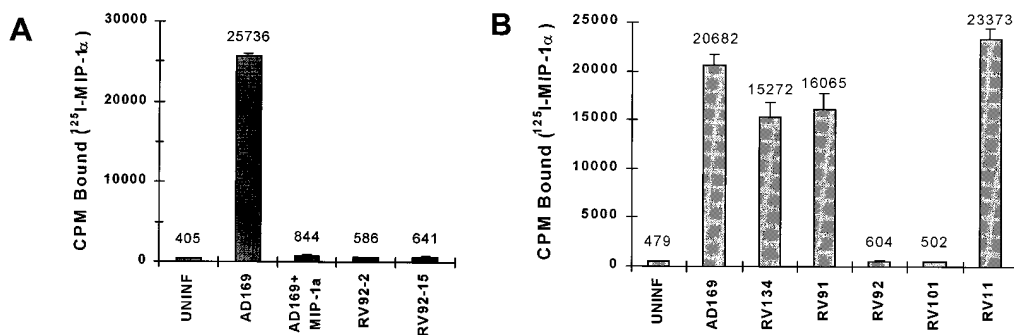


Figure 6. MIP-1 α binding by cells infected with HCMV mutants. Uninfected or HCMV-infected HFF cells (multiplicity of infection 2.5) were analyzed for their ability to bind ¹²⁵I-labeled MIP-1 α at 72 h pi. RV92-2 and RV92-15 are independent isolates containing the identical US28 deletion. In some experiments, excess unlabeled MIP-1 α (200 nM final concentration) was used (AD169+MIP-1 α). cpm of bound radiolabeled MIP-1 α are given above each bar.

are undergoing continual binding and rapid internalization of the chemokines.

To examine the possibility that chemokines released from HCMV-infected cells may be subsequently bound by HCMV-encoded receptors, several experiments were performed. The first experiment was based on the observation

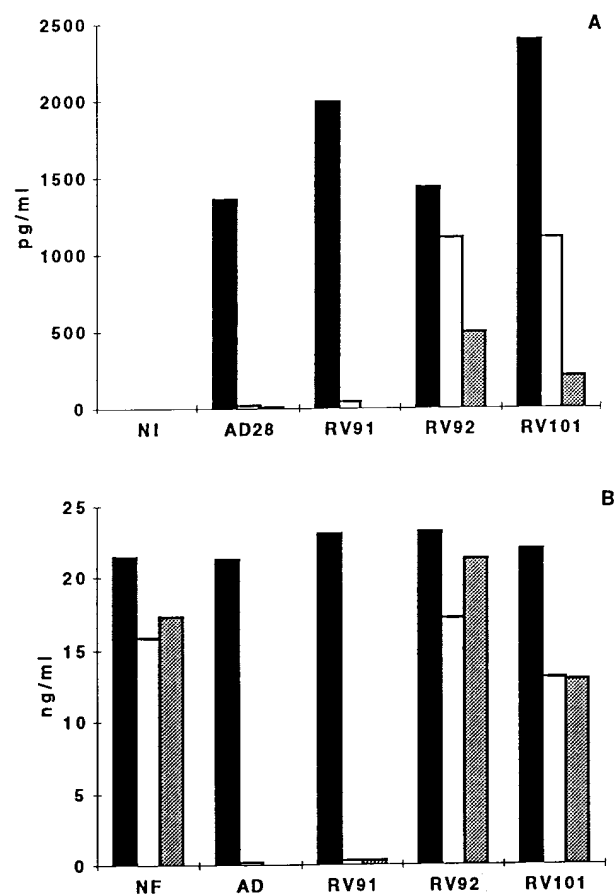


Figure 7. (A) RANTES expressed in picogram/milliliter or (B) MCP-1 expressed in nanogram/milliliter were measured by ELISA in supernatants collected at 0–24 h (black bars), 24–48 h (white bars), and 48–72 h (gray bars) after infection from either uninfected cells (NF) or cells infected with wild-type or mutant HCMVs: AD (wild-type), RV91 (Δ US27), RV92 (Δ US28), RV101 (Δ US27/ Δ US28). At each interval, cells were washed and re-fed growth medium.

that chemokine expression can be induced by HCMV infection of fibroblasts and accumulates in the extracellular medium at 48–72 h pi of cells infected with US28 mutants (Fig. 7). Conditioned medium collected at 72 h pi from cells infected with US28-negative mutants or US28-positive virus infected cells as control was used as a cold competitor in an MIP-1 α -binding assay to wild-type HCMV-infected cells (Table 3). Relative to fresh medium, media conditioned on cells infected with US28-negative mutants inhibited 48% of total MIP-1 α binding. In contrast, media conditioned on US28-positive cells inhibited MIP-1 α binding by <5%.

In a second experiment, exogenous rRANTES was added to HCMV-infected HFF cells to see if it would be removed from the medium. At 48 h pi, medium was collected and cells were washed with PBS, then treated with a low pH glycine buffer to strip the membrane of any chemokine (34). Exogenous rRANTES was then added to culture medium and cells were incubated for different times at 37°C before measuring the amount of RANTES

Table 3. Adsorption of MIP-1 α from Conditioned Media

| Conditioned medium source | Cpm MIP-1 α bound (standard deviation) | Percentage of reduction of MIP-1 α binding |
|---------------------------|---|---|
| None (fresh medium) | 27,164 (1,750) | None |
| US28-positive cells | 25,962 (4,362) | 4.5 |
| US28-negative cells | 14,090 (1,802) | 48.2 |

Target cells in the MIP-1 α binding assay were HCMV strain AD169-infected HFF cells at 72 h pi. The binding assay was done as described in Materials and Methods using 0.25 nM of radiolabeled MIP-1 α per well, except that 0.125 ml of conditioned medium replaced an equal volume of cold binding buffer. No attempt was made to remove chemokines from the infected cell membranes before the initiation of the binding assay. Each data point represents the mean and standard deviation derived from three to four plates, as follows: US28-positive infected cell-conditioned medium was from one plate each of HFF cells infected with wild-type strains AD169, RV134, RV91, and RV11. US28-negative infected cell-conditioned medium was from cells infected with RV92 (one plate) and RV101 (two plates).

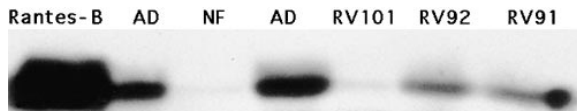


Figure 8. Fibroblasts infected for 48 h with wild-type (AD) or mutant HCMVs (RV101, RV92, RV91) were washed with PBS, incubated for 3 min at room temperature with low pH glycine buffer to strip membranes of chemokine, and then incubated with biotinylated RANTES (100 nM) for 3 h at 37°C. Cells were then washed again with PBS, treated with low-pH glycine buffer, trypsinized, and extracted in a high salt buffer. Extracts were separated in 15% SDS-PAGE and transferred to nitrocellulose. After saturation with 3% gelatin/1% BSA in PBS/0.1% Tween 20, blots were incubated with peroxidase-labeled streptavidin (1:400) and developed using a chemiluminescence technique for 5 s. RANTES-B (Rantes-B, 125 ng) was run in parallel. AD = infected with wild-type HCMV; RV91 = infected with Δ US27 mutant HCMV; RV92 = infected with Δ US28 mutant HCMV; RV101 = infected with Δ US27/ Δ US28 mutant HCMV; NF = uninfected cell extract. Representative of two experiments.

remaining in the medium. Incubation of HCMV-infected fibroblasts with 600 pg/ml of rRANTES for 30 min or 3 h resulted in a 12 and a 72% reduction, respectively, of the exogenously added chemokine. Similar experiments were carried out on cells infected for 72 h. An average (two experiments run in duplicate) of 59% of added rRANTES disappeared from the medium of infected cells after a 3-h adsorption period at 37°C. In all experiments, RANTES was not detected in the medium sampled before starting the adsorption period (i.e., before the addition of exogenous rRANTES). Taken together, these experiments indicate that chemokines synthesized and released from HCMV-infected cells can bind to, at least, the US28-encoded receptor on the surface of these cells.

It is known that when a chemokine contacts its receptor, both are internalized and ligand is released from the receptor, which then recirculates to the cell surface (42). To determine whether extracellular RANTES was being internalized from the cell surface after receptor binding, uninfected cells or HCMV-infected cells (at 48 h pi) were incubated with RANTES-B for 3 h at 37°C, washed to remove cell surface (noninternalized) chemokine, then analyzed by immunoblot using peroxidase-labeled streptavidin, as described in Materials and Methods (Fig. 8). The results demonstrate that RANTES-B was efficiently internalized by cells infected with wild-type HCMV, less so by cells infected with the mutants RV91 and RV92, and not at all by RV101-infected cells or uninfected fibroblasts.

Discussion

After primary infection, HCMV, like other herpesviruses, establishes itself in a latent or persistent state and resides in its host for the remainder of the host's lifetime (43). To facilitate this state, HCMV has several means designed seemingly to avoid elimination of infected cells by the immune surveillance system. HCMV encodes at least four genes whose products play a role in the downregulation of surface expression of HLA class I molecules (9, 10, 18–23).

In addition, HCMV infection can interfere with normal cytokine production (44) and upregulate production of such lymphostatic factors as TGF- β (45) and IL-1Ra (46).

However, the HCMV genome also carries three genes with homology to CCRs (UL33, US27, and US28; reference 39). The role of these receptors in the life cycle of human HCMV in vivo has not yet been determined. HCMV UL33 was shown to be a virion envelope protein, but its function remains unknown (27). The murine cytomegalovirus homologue of HCMV UL33, designated M33 (47), may play a role in the establishment of latency of murine HCMV in salivary glands (48). Unfortunately, murine HCMV does not possess homologues of US27 and US28. Of the HCMV CCR homologues, only US28 has been shown to be functional to date. In transfected mammalian cells, US28 mobilizes Ca²⁺ in response to RANTES, MIP-1 α , and MCP-1 (29, 30); in HeLa and astrocytoma cells, it can act as a coreceptor for HIV entry (49). However, these studies do not shed light on the function of US28 in the context of HCMV infection.

In the context of HCMV-infected cells, we show here that the product of the HCMV US28 gene plays a prominent role in binding and sequestering of extracellular CC chemokines. Through the use of specific HCMV gene disruption/deletion recombinant mutants, the US28-encoded receptor was shown to be the only MIP-1 α binding receptor expressed in HCMV-infected cells. Cells infected with a US27 mutant (RV91) or a UL33 mutant (RV11), but not a US28 mutant (RV92) or a US27+US28 mutant (RV101), retained ability to bind the CC chemokine MIP-1 α . In conjunction with our inability to detect CCR polyadenylated transcripts in infected cells, this observation effectively rules out the possibility that HCMV infection induces expression of cellular MIP-1 α receptors. In addition to MIP-1 α binding in HCMV-infected cells, the US28-encoded receptor binds other CC chemokines, including MIP-1 β , RANTES, and MCP-1, consistent with the binding observed in US28-transfected cells (29, 30). We have extended these observations in that the US28-encoded receptor also binds MCP-3, but not MCP-2, both CC chemokines. Interestingly, at the amino acid level MCP-2 is highly homologous to MCP-1 and MCP-3 (62 and 60%, respectively; reference 41).

Cell surface expression of the US28-encoded receptor results in the depletion of CC chemokines, especially RANTES and MCP-1, from the medium of HCMV-infected cells. Cells infected with mutants that expressed the other putative chemokine receptors, US27 or UL33 (i.e., RV101 and RV92), but lacked US28 expression, failed to efficiently deplete chemokines from the medium (Fig. 7). Furthermore, cells infected with viruses that expressed US28 internalized exogenous RANTES, whereas uninfected cells or cells infected with RV101, deleted of both US27/US28, failed to internalize added RANTES (Fig. 8). Taken together, these data indicate that UL33, which is present and transcribed at wild-type levels in cells infected with RV101 (data not shown), does not play a role in the sequestration of RANTES or MCP-1.

Remarkably, the ability to internalize RANTES did not strictly correlate with US28 expression. Cells infected with US28 mutant RV92 or US27 mutant RV91 internalized RANTES, although to a lesser extent than cells infected with wild-type HCMV. Although it is clear from our experiments that the US28-encoded receptor functions to bind and internalize CC chemokines in HCMV-infected cells, the US27-encoded receptor may also contribute to these processes. Thus, it is possible that US28 is not the only HCMV-encoded receptor that binds and internalizes RANTES. The US27-encoded receptor may also bind/internalize RANTES, but has differential affinity for MIP-1 α (i.e., the US28-encoded receptor, but not the US27-encoded receptor, binds MIP-1 α). We are currently investigating the possibility that the US27-encoded receptor is a low affinity receptor for some CC chemokines.

We propose that a role for the chemokine receptors expressed in HCMV-infected cells may be the ability to sequester chemokines from the extracellular milieu. We demonstrated recently (38), and confirm here, that chemokines accumulate intracellularly in HCMV-infected cells. This appears to be partially due to continual internalization from the exterior, as shown here, since in HCMV-infected cells (a) exogenously added RANTES disappeared from cell medium and (b) added biotinylated RANTES was found intracellularly. We cannot exclude the possibility that endogenously produced chemokines may also be sequestered intracellularly without ever being secreted. When exposed to ligand, the US28-encoded receptor in transfected cells can initiate a transmembrane signal that results in second messenger signaling, including calcium flux (29, 30). This may also occur in HCMV-infected cells and alter cell physiology, thereby affecting replication of the virus. However, the presence or absence of chemokines had no detectable effect on the growth properties of HCMV wild-type in fibroblasts compared with US27+US28 mutant RV101 (Jones, T.R., unpublished data). Additionally, Pertussis

toxin, which interferes with signaling of some cellular chemokine receptors (41, 50), likewise had no effect on growth of these viruses (Jones, T.R., unpublished data).

Induction and disappearance of RANTES are two separable phenomena. Induction occurs independently of an active CMV genome upon contact/penetration of virus into fibroblasts, as illustrated by the fact that UV-inactivated virus induces RANTES production. Induction could therefore be due to contact of viral elements with cell membranes and/or introduction of viral proteins and DNA into the cell. Hence, even an abortive infection in the absence of viral genome expression might induce production of RANTES, which could in turn induce recruitment of lymphocytes.

On the other hand, disappearance of RANTES as infection progresses does depend on viral genome expression, since it does not occur when cells are infected with UV-inactivated virus. Hence, when CMV genome expression progresses and US28 and US27 are encoded, CC chemokines, whether induced by virus contact (RANTES) or not (MCP-1), are sequestered from the infected cell environment. Such sequestration could prevent not only lymphocyte recruitment, but also stimulation of effector mechanisms of lymphocytes in the neighborhood of infected cells. The capacity of HCMV infected cells to sequester CC chemokines could potentially perturb local immune responses by altering their concentration in the proximal extracellular milieu. CC chemokines have been shown to play a role in inflammation and infiltration by lymphocytes, monocytes, eosinophils, and basophils at disease sites (41, 51). They are also important in activating the cytotoxic potential of CD8⁺ lymphocytes (52, 53) and NK cells (1). Thus, this sequestration could have a negative effect on the attraction of leukocytes to sites of CMV infection and on the immune function of leukocytes in the environment of infected cells. Hence, this CMV strategy would help the virus to go unnoticed and thereby escape immune surveillance.

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