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A Novel Link between Stress and Human Cytomegalovirus (HCMV) Infection: Sympathetic Hyperactivity Stimulates HCMV Activation

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Recently, inflammatory mediators such as TNFα were identified as triggering active human cytomegalovirus (HCMV) infection. Here, we demonstrate that a highly stressful event in the absence of systemic inflammation, as observed in patients with acute myocardial infarction, leads to the development of an active HCMV infection in latently infected patients. Elucidating the molecular mechanism of virus activation, we could show that catecholamines directly stimulate the HCMV immediate-early (IE) enhancer/promoter in monocytic cells via β-2 adrenergic receptors. Subsequent activation of the cAMP/PK-A-signaling pathway results in enhanced synthesis and binding of the transcription factor CREB-1/ATF-1 to the cAMP-responsive elements within the IE enhancer. Epinephrine also enhanced HCMV gene expression in infected THP-1 cells by about 50% in three of four experiments. These data suggest that HCMV, like HSV-1 and VZV, can be (re)activated under stress conditions.

INTRODUCTION

Active infections with the human cytomegalovirus (HCMV) play an important role in the morbidity and case fatality of organ and bone marrow transplant recipients and AIDS patients. In addition to the risk of those patients developing classical signs of acute and chronic HCMV disease (e.g., fever, leukopenia, pneumonitis, hepatitis, retinitis), active HCMV infections may be associated with acute and chronic graft failure (Grattan *et al.,* 1989; Reinke *et al.,* 1994) and secondary immune deficiency (Fishman and Rubin, 1989). However, their pathological significance seems not to be restricted to immunocompromised patients.

Active HCMV infections result mostly from reactivation of latent virus harbored by more than 60% of adults. Granulocyte/monocyte progenitors in the bone marrow were identified as at least one site of HCMV latency (Taylor-Wiedeman *et al.,* 1991; Mendelson *et al.,* 1996; Kondo *et al.,* 1996). Very recently reactivation of latent HCMV from blood monocytes and latently infected granulocyte/monocyte progenitors has been described (Söderberg-Naucler *et al.,* 1997; Hahn *et al.,* 1998). Expression of the major immediate-early (IE) proteins IE-1 and IE-2 was shown to be critical for transition from latency to productive infection (Iskenderian *et al.,* 1996; Greaves and Mocarski, 1998). Expression of these proteins is controlled by the major IE enhancer/promoter whose activity depends on both viral and cellular factors/proteins (for review see Meier and Stinski, 1996).

Recently, by clinical studies and *in vitro* experiments we have demonstrated that systemic inflammation, in particular TNFα release, may be an important cofactor for HCMV (re)activation in transplant patients as well as nonimmunosuppressed patients with septicemia or other inflammatory diseases through up-regulation of the IE enhancer/promoter activity by NF-κB (Stein *et al.,* 1993; Döcke *et al.,* 1994; Fietze *et al.,* 1994; Prösch *et al.,* 1995, 1998b). These observations were confirmed by others demonstrating TNFα-mediated HCMV reactivation in latently infected monocyte/granulocyte progenitor cells (Hahn *et al.,* 1998) as well as *ex vivo* reactivation of MCMV from bone marrow myelomonocytes that were isolated from latently infected animals (Henry, 1999).

However, in humans, active HCMV infection has been also observed in patients without any signs of a systemic inflammation. HCMV-associated encephalitis and colitis have been reported in nonimmunosuppressed patients (Studahl *et al.,* 1992; Blair *et al.,* 1992; Maignan *et al.,* 1992; Prösch *et al.,* 1998a). Furthermore, increased prevalence of HCMV seropositivity and higher serum anti-HCMV IgG levels were reported in nonimmunosuppressed, nonmalignant patients with peptic ulcer and nonulcer dyspepsia as well as in healthy people under physical stress (Glaser *et al.,* 1985; Archimandritis *et al.,* 1992). An association was found between stress-produc-

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ing events and the number of HCMV DNA-positive urine and leukocyte samples (Toro and Ossa, 1996). These data suggest that stressful conditions may induce molecular events that promote (re)activation of HCMV expression and replication, but the mechanism(s) is still unknown.

A relationship between physical or psychic stress and reactivation of other members of the herpesvirus family, e.g., herpes simplex virus (HSV), is well established (for reviews see Steiner, 1996; Turner and Jenkins, 1997). Stress induces the release of several mediators such as catecholamines, glucocorticoids, and thyroid hormones from the synaptic nervous system and endocrine glands. In animal models and clinical studies it has been shown that stressors such as epinephrine, cyclophosphamide, transient hyperthermia, UV light, and glucocorticoids induce (re)activation of HSV (Perna *et al.,* 1987; Steiner, 1996; Turner and Jenkins, 1997). However, the molecular mechanism(s) underlying these processes is poorly understood.

The aim of the present study was to investigate the relation between stress and (re)activation of HCMV infection in humans. In a prospective clinical study we investigated the incidence of active HCMV infection in patients with acute myocardial infarction—a highly stressful event that was shown to be associated with strongly elevated catecholamine but normal TNFα plasma levels. By *in vitro* experiments a direct stimulatory effect of catecholamines on the activity of the HCMV IE enhancer/promoter and on viral gene expression could be identified.

RESULTS

Catecholamine release in myocardial infarction is associated with HCMV activation

In a prospective clinical study, 10 patients with acute myocardial infarction were monitored for plasma epinephrine and norepinephrine levels as well as for parameters of HCMV infection from intensive care unit admission up to 14 days after the acute event. Catecholamine plasma concentrations were enhanced at admission and normalized on day 4 after myocardial infarction (Fig. 1). There was a positive correlation between epinephrine and norepinephrine plasma levels, which was strongest on day 1 ($r = 0.81$, $P = 0.005$; Pearson coefficient with two-tailed significance levels).

Nine of the 10 patients (90%) had IgG anti-HCMV antibodies in serum. The levels increased in 5 of 9 patients during the 2-week follow-up. Specific IgM was never detectable. None of the patients with myocardial infarction established HCMV disease-related symptoms within the observation period of 14 days. However, all patients developed positive HCMV DNA PCR from peripheral blood mononuclear cells (PBMC) during the first week. One day after admission, HCMV DNA was already

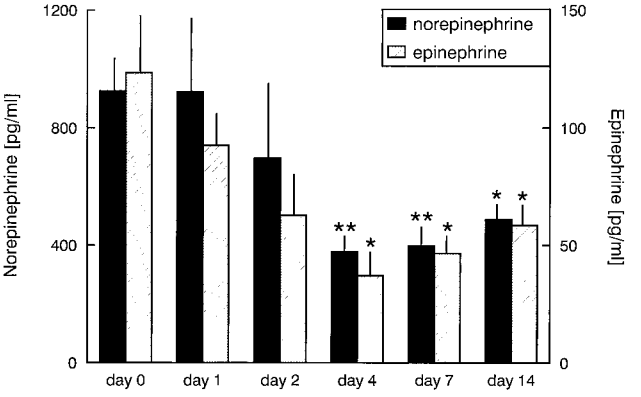

FIG. 1. Myocardial infarction is associated with sympathetic hyperactivity. Norepinephrine and epinephrine serum levels were determined by HPLC in patients with acute myocardial infarction (normal ranges 165–460 and 30–90 pg/ml, respectively). Data are given as means ± SEM. *$P < 0.05$, **$P < 0.01$ vs admission, Wilcoxon test.

detectable in 5 of 8 patients tested, and within the first 4 days all 10 patients were PCR-positive including the one patient who was (and remained) seronegative. Thereafter, the prevalence of positive HCMV DNA PCR dropped again; on day 14 only 2 of 7 patients (3 patients had been discharged from the hospital before) were still positive.

Moreover, using immunocytology, on day 1, in 3 of the 10 patients at least one cell per 10,000 PBMC was found to be positive for HCMV IE or early antigens (Fig. 2). The mean frequency of HCMV antigen-positive cells, as assessed by summation of the counts for three different antibodies, was as low as 1.62 ± 0.92 HCMV antigen-positive cells per 10,000 PBMC (mean ± SEM), which was comparable to this level in healthy probands (0.64 ± 0.21 per 10,000 PBMC, $P > 0.05$ in a Mann–Whitney U test). On day 4, 8 patients already had HCMV antigen-positive PBMC with a slightly increased frequency of 5.66 ± 2.04 per 10,000 cells ($P < 0.05$ vs day 1; $P < 0.001$ vs controls). Seven days after the acute myocardial infarction all 10 patients had developed a strong HCMV antigenemia with a mean frequency of 91.47 ± 25.61 HCMV antigen-positive cells per 10,000 PBMC ($P < 0.01$ vs day 1 and vs day 4). Interestingly, this peak frequency of HCMV antigen-positive cells was significantly correlated to postinfarction peak plasma levels of both norepinephrine ($r = 0.78$, $P = 0.005$) and epinephrine ($r = 0.63$; $P = 0.039$). Moreover, the highest frequencies of HCMV antigen-positive cells (189 and 275 per 10,000 PBMC, respectively) were found in 2 patients who presented with cardiogenic shock at admission and needed catecholamine infusions.

Two weeks after the acute event, HCMV antigenemia markedly declined in the remaining seven patients investigated although the number of positive cells was still significantly elevated (4.51 ± 1.63 per 10,000 PBMC) in comparison to day 1 ($P < 0.05$) and healthy probands ($P < 0.001$).

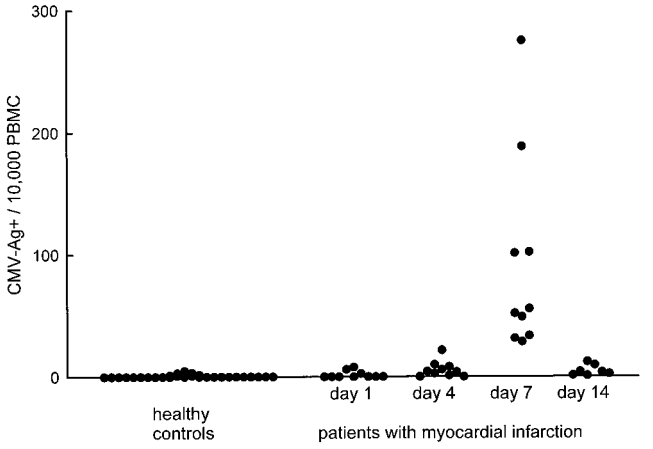

FIG. 2. Myocardial infarction triggers HCMV antigenemia. HCMV antigen-positive cells were determined for 10 patients with acute myocardial infarction. The overall frequency of HCMV antigen-positive PBMC as determined by the APAAP technique using mAb recognizing HCMV IE-1 antigen, pp65 early matrix protein, and p52 early DNA binding protein was calculated by summarizing those positive for the different antigens. Individual values are shown. The mean frequency (±SEM) of antigen-positive cells per 10,000 PBMC for all patients was 1.62 ± 0.92 on day 1; 5.66 ± 2.04 on day 4 ($P < 0.05$); 91.47 ± 25.61 on day 7 ($P < 0.01$); and 4.51 ± 1.63 on day 14 ($P < 0.05$ vs day 1; Wilcoxon test). For comparison, the results from 32 seropositive blood donors are given (mean frequency of HCMV antigen-positive cells: 0.64 ± 0.21 per 10,000 PBMC). The frequencies of HCMV antigen-positive cells in our patient group were comparable to those in healthy controls on day 1 ($P > 0.05$) but significantly enhanced on days 4, 7, and 14 after myocardial infarction ($P < 0.001$, $P < 0.0001$, and $P < 0.001$ in a Mann–Whitney *U* test).

When looking at the different HCMV antigens, at all time points the highest frequency of positive cells was found for the IE-1 antigen. On day 7 after myocardial infarction, however, the frequencies for all HCMV antigens investigated were significantly enhanced in comparison to day 1 (for IE-1 antigen, 56.5 ± 12.9 vs 1.7 ± 0.5 antigen-positive cells per 10,000 PBMC; for pp65 antigen, 20.5 ± 4.7 vs 0.6 ± 0.4 antigen-positive cells per 10,000 PBMC; for p52 antigen, 15.3 ± 4.1 vs 0.6 ± 0.4 antigen-positive cells per 10,000 PBMC). The majority of infected cells were monocytes; however, positive lymphocytes were detected too (not shown).

Catecholamines stimulate the HCMV IE enhancer/promoter in monocytic cells

To verify a direct role of catecholamines in stimulation of active HCMV infection, we studied their influence on HCMV IE enhancer/promoter activity in the monocytic cell line THP-1, which is semipermissive for HCMV (Weinshenker *et al.,* 1988) and may serve as a model of actively infected monocyte/macrophages *in vivo*.

As demonstrated in Fig. 3A, the catecholamines epinephrine and norepinephrine, but not the stress hormones thyroxine and ACTH, caused a strong stimulation of the IE enhancer/promoter-controlled CAT expression in pRR55-transfected THP-1 cells. Marginal stimulation was also observed in corticosteroid-treated cells. The stimulatory effect of epinephrine and norepinephrine was concentration-dependent (Fig. 3B). Catecholamines also significantly stimulated the IE enhancer/promoter activity in transfected freshly isolated monocytes from buffy coat of healthy blood donors by factor of 2.5 to 3 (data not shown).

Catecholamine stimulation is mediated via the β-2-adrenoreceptors and involves protein kinase A

To learn more about the mechanisms of catecholamine-dependent stimulation of the IE enhancer/promoter, pRR55-transfected THP-1 cells were preincubated with increasing concentrations of the β-adrenergic re-

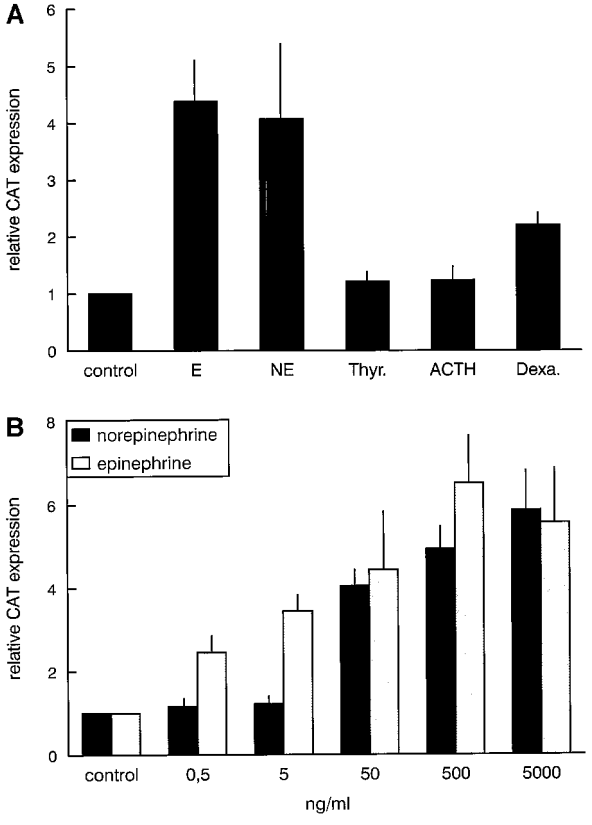

FIG. 3. Influence of stress hormones on the HCMV IE enhancer/promoter activity in pRR55-transfected THP-1 cells. (A) Selective stimulation by catecholamines. After transfection with plasmid pRR55, THP-1 cells were incubated in the absence (control) or in the presence of 500 ng/ml epinephrine (E), norepinephrine (NE), thyroxine (Thyr.), ACTH, or dexamethasone (Dexa.). Cells were harvested after 40 h for CAT assay. The graph represents the relative CAT expression rate of four independent experiments (means ± SEM); significance was demonstrated in Friedman test ($P < 0.05$). (B) Dose-dependent effects of epinephrine and norepinephrine. pRR55-transfected THP-1 cells were incubated with different concentrations of epinephrine or norepinephrine. The graph represents mean (±SEM) values of relative CAT expression rates obtained in four independent experiments. For both epinephrine and norepinephrine, significant dose-dependent differences were demonstrated by the Friedman test ($P < 0.01$).

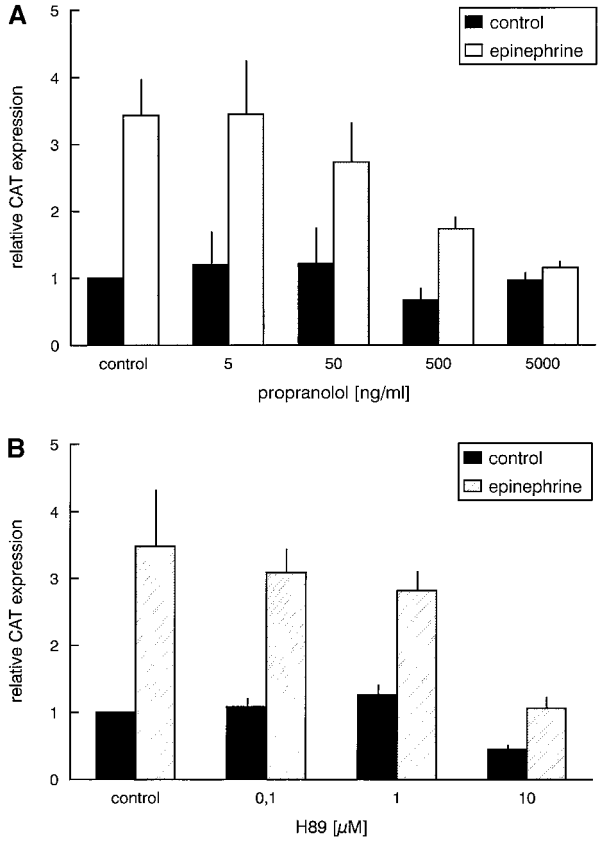

FIG. 4. The catecholamine effect is mediated through β-2-adrenoreceptors and protein kinase A activation. THP-1 cells transfected with the plasmid pRR55 were incubated in the absence (control) or in the presence of epinephrine (500 ng/ml) and different concentrations of the β-1/β-2-adrenoreceptor blocker propranolol (A) or the protein kinase A inhibitor H89 (B). Propranolol or H89 was added at time zero after transfection and 60 min before addition of epinephrine. The mean values (±SEM) of relative CAT expression determined in four independent experiments (propranolol) and three independent experiments (H89) are presented. For both agents, significant dose-dependent differences in CAT expression were demonstrated in the epinephrine group ($P < 0.05$, Friedman test).

ceptor blockers propranolol and metoprolol. Whereas the selective β-1-receptor antagonist metoprolol had no effect, propranolol, which blocks both β-1- and β-2-adrenergic receptors, completely abrogated the stimulatory effect of epinephrine (Fig. 4A) and norepinephrine (data not shown).

The majority of effects downstream from the β-2-adrenergic receptor are known to be mediated by the cAMP/protein kinase (PK)-A-dependent signaling pathway. Therefore, we tested the effect of the PK-A inhibitor H89 on epinephrine/norepinephrine-dependent stimulation. Preincubation of pRR55 transfected THP-1 cells with H89 (Fig. 4B) but not with the PK-C inhibitor H7 (data not shown) reduced the stimulatory effect of epinephrine on IE promoter activity in a dose-dependent manner. High but nontoxic concentrations of H89 (but not of H7) also down-regulated the basal activity of the HCMV IE enhancer/promoter, which had been shown to depend mainly on the activity of the CREB-1/ATF-1 transcription factor (Stamminger *et al.,* 1990).

Catecholamines target a CRE-containing 19-bp sequence motif of the HCMV IE enhancer

The enhancer of the HCMV IE promoter contains five 19-bp repetitive sequence motifs each containing a cAMP-responsive element (CRE), which binds the transcription factor CREB-1/ATF-1 (Hunnighake *et al.,* 1989; Meier and Stinski, 1996). To prove whether the stimulatory effect of catecholamines is mediated via the known CRE in the 19-bp sequence motifs of the IE enhancer, THP-1 cells were transfected with plasmid constructs containing the minimal IE promoter alone (pUPCAT) or in combination with four in-tandem copies of the 19-bp sequence motif with the CRE (p4-19PCAT). Control transfections were performed with plasmid constructs containing the minimal IE promoter and four in-tandem copies of the 18-bp sequence motif including a NF-κB-binding site (p4-18PCAT), the 17-bp sequence motif (p4-17PCAT), or the 21-bp sequence motif (p4-21PCAT).

Epinephrine had no effect on CAT expression in cells transfected with plasmids containing the minimal promoter alone or in combination with either the 17- or the 21-bp sequence motifs (data not shown). Only marginal stimulation was observed in p4-18PCAT transfected cells (Fig. 5). In contrast, a very strong stimulation of CAT expression occurred in those cells transfected with the plasmid p4-19PCAT as well as in cells transfected with the plasmid p4-18/4-19PCAT (Fig. 5). As for the complete IE enhancer/promoter, stimulation via the CRE-containing 19-bp sequence motifs was decreased by H89 (70% inhibition of the epinephrine-dependent stimulation) but not by H7 (0% inhibition) and could be antagonized by

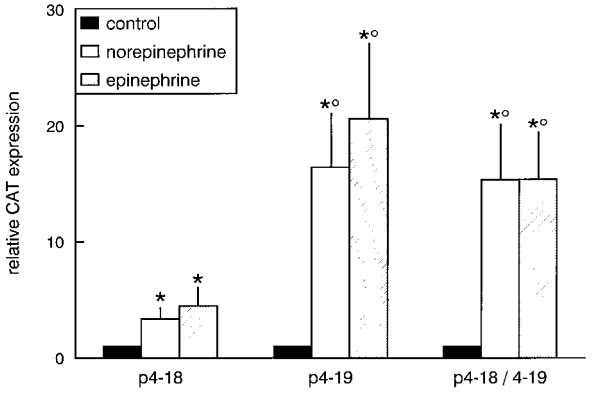

FIG. 5. Catecholamines target the 19-bp sequence motif of the HCMV IE enhancer. THP-1 cells were transfected with the plasmid constructs p4-18PCAT, p4-19PCAT, or p4-18/4-19PCAT and incubated in the absence (control) or in the presence of 500 ng/ml epinephrine or norepinephrine. Mean (± SEM) values of relative CAT conversion rates of five independent experiments are presented. *$P < 0.05$ vs respective control, Wilcoxon test; °$P < 0.05$ vs p4-18CAT, Wilcoxon test.

propranolol (90% inhibition), indicating that the CREs indeed mediate the catecholamine-induced stimulation of the IE enhancer/promoter and this occurs via the β-2-adrenergic receptor.

Catecholamines increase synthesis of CREB-1 and the binding activity of CREB-1/ATF-1 to the CREs

In an electrophoretic mobility shift assay (EMSA), a significantly increased protein-binding activity of the 19-bp sequence motif was evident in nuclear extracts from epinephrine- or norepinephrine-treated THP-1 cells (Fig. 6A, lanes 3 and 1, respectively) compared to the nuclear extract from untreated THP-1 cells (lane 2). Binding of the protein(s) was highly specific and could be completely competed by preincubation of the nuclear extract with unlabeled 19-bp oligonucleotide (data not shown) and an oligonucleotide concerning the CRE consensus sequence (Fig. 6A, lanes 4, 5, and 10). Moreover, the bound protein complex reacted in supershifts with antibodies specific for the transcription factors ATF-1 and CREB-1 (Fig. 6A, lanes 6–9, 11, and 12) but not with antibodies against NF-κB p65 and AP-1/c-jun (data not shown).

In Western blot analysis using nuclear extracts from untreated and catecholamine-treated THP-1 cells and a polyclonal antiserum specific for CREB-1 we observed an increased amount of CREB-1 protein (p43) in catecholamine-treated cells (Fig. 6B, lanes 7 and 8) compared to untreated cells (Fig. 6B, lanes 5 and 6). To demonstrate equivalent loading of nuclear proteins a Coomassie blue-stained gel is shown in Fig. 6B, lanes 2–4.

Epinephrine enhances HCMV gene expression in infected THP-1 cells

Next we wondered whether epinephrine stimulates HCMV gene expression in virus-infected monocytic cells. THP-1 cells were infected with the recombinant HCMV RC256 expressing β-galactosidase and proven to have β-galactosidase activity 72 h postinfection using the *O*-nitrophenyl-β-D-galactopyranoside method (MacGregor *et al.,* 1991). In three of four independent experiments 500 ng/ml epinephrine enhanced β-galactosidase expression by about 50% as shown in Table 1.

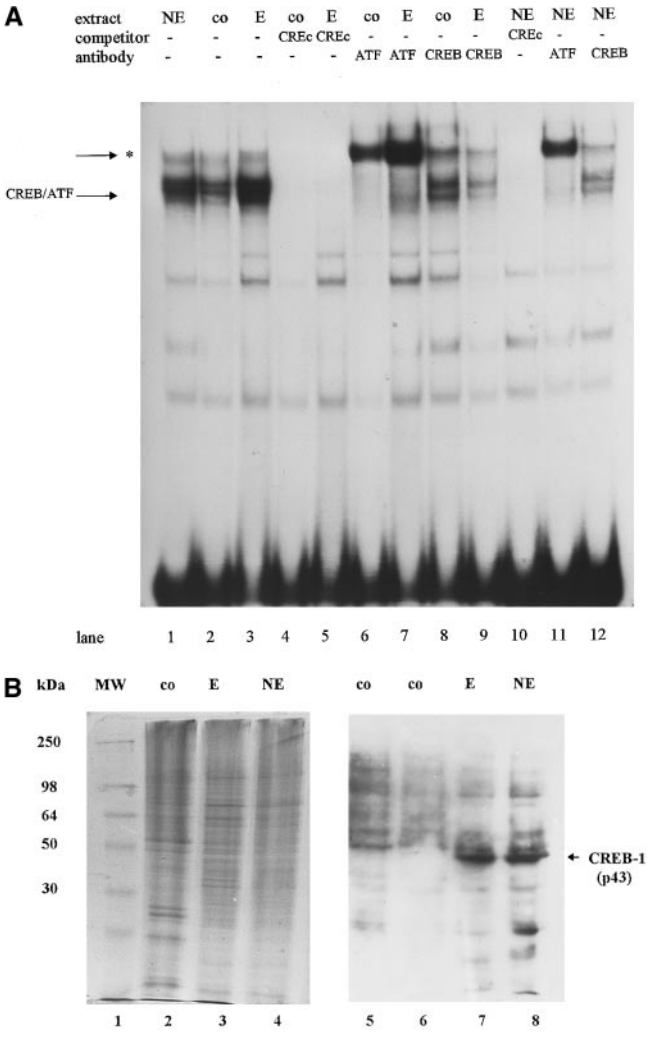

FIG. 6. (A) Catecholamines increase the binding activity of CREB-1/ATF-1 to the CREs of the 19-bp sequence motif of the HCMV IE enhancer. Radiolabeled oligonucleotides with the 19-bp sequence motif of the IE enhancer were incubated with nuclear extracts of THP-1 cells incubated for 12 h in the absence (co) or in the presence of 500 ng/ml epinephrine (E) or norepinephrine (NE). Specificity of the binding was verified by preincubation (5 min) of the nuclear extract with a 50 molar excess of an oligonucleotide containing the CRE consensus sequence (CREc) (Santa Cruz Biotechnology, Santa Cruz, CA). For identification of the bound protein complex, 1.5 μl of the antibodies specific for CREB-1 (CREB) and ATF-1 (ATF) (Santa Cruz Biotechnology) was added to the reaction mix. The autoradiograph shows one representative experiment that was repeated four times. *Slower migrating protein complex after incubation with the appropriate antibody. (B) Epinephrine and norepinephrine enhance CREB-1 synthesis in THP-1 cells. Nuclear protein extracts from untreated (co), epinephrine-treated (500 ng/ml; E), or norepinephrine-treated (500 ng/ml; NE) THP-1 cells were analyzed for CREB-1 (p43) protein amounts by Western blot (lanes 5–8). For loading control a gel with identical probes was stained with Coomassie blue (lanes 2–4). In lane 1, a protein molecular weight standard is shown. The figure demonstrates one representative blot that was repeated three times.

DISCUSSION

Recently, TNFα was identified as an important risk factor for (re)activation of HCMV infection in several patient groups (Döcke *et al.,* 1994; Fietze *et al.,* 1994). By *in vitro* experiments we could show that TNFα up-regulates the activity of the major HCMV IE enhancer/promoter in premonocytic cells (Stein *et al.,* 1993; Prösch *et al.,* 1995, 1998b). Accordingly, TNFα was shown to mediate reactivation of HCMV in latently infected progenitor cells (Hahn *et al.,* 1998).

The present study defines an additional novel risk factor of HCMV (re)activation and demonstrates a close link between stress-related catecholamine response and

TABLE 1

Influence of Epinephrine on HCMV Gene Expression in RC256-Infected THP-1 Cells

Experiment No.	Control β-gal-activity (Units)	Epinephrine (500 ng/ml) β-gal-activity (Units)	Stimulation factor
I	0.52	0.78	1.5
II	0.48	0.32	0.7
III	2.40	3.90	1.63
IV	1.48	2.18	1.5

Note. THP-1 cells (10^6) were infected with HCMV strain RC256 (m.o.i. = 1) and cultivated in the absence or in the presence of 500 ng/ml epinephrine. After 72 h, cell extracts were prepared and measured for β-galactosidase activity by the ONPG method. Data from four independent experiments are shown.

HCMV infection. Within a few days following the highly stressful event of acute myocardial infarction, all 10 patients developed HCMV antigenemia, particularly in monocytes, which are a site of viral reactivation from latency (Söderberg-Naucler *et al.,* 1997). Although myocardial infarction induces a local inflammation in the ischemic tissue, we could not find a significant increase of TNFα plasma levels in these patients (data not shown). Therefore, it is unlikely that the active HCMV infection was due to systemic inflammation.

There was a strong correlation, however, between catecholamine plasma peak levels in the early postinfarction period and the frequency of HCMV antigen-positive cells on day 7. As the half-life of monocytes in the peripheral blood is less than 24 h, we can presume that catecholamines triggered HCMV protein synthesis in the monocytic bone marrow progenitor cells. A similar delay between the triggering event and appearance of HCMV antigenemia was observed following OKT3-induced TNFα release in transplant patients (Fietze *et al.,* 1994), suggesting that bone marrow progenitor cells are an important pool of HCMV at latency/persistence (Taylor-Wiedeman *et al.,* 1991; Mendelson *et al.,* 1996; Kondo *et al.,* 1996).

Our *in vitro* studies support for the first time a strong relation between catecholamines and HCMV activity in monocytic cells. In the presence of epinephrine, viral gene expression (as measured by β-galactosidase expression) in RC256-infected THP-1 cells representing mature monocytes was increased by about 50% in three of four experiments. In transient transfection experiments using the same cell line, catecholamines directly stimulated the HCMV IE promoter via the β-2-adrenergic receptor.

Similarly, HSV-1 reactivation by hyperthermia involves this receptor also (Gebhardt and Kaufman, 1995). Following binding of the hormone, a signal cascade is triggered involving stimulation of PK-A and enhanced synthesis of the transcription factor complex CREB-1/ATF-1, which specifically binds to the CRE-containing 19-bp repetitive sequence motifs of the IE enhancer/promoter (Hunnighake *et al.,* 1989). The transcriptional activity of CREB-1/ATF-1 requires phosphorylation at serine 133, which is known to be also regulated by the cAMP-dependent PK-A (Gonzalez *et al.,* 1989). Thus, in addition to the demonstrated increased synthesis, enhanced phosphorylation of the constitutively expressed transcription factor complex CREB-1/ATF-1 might be responsible for the stimulation of HCMV IE promoter-controlled transcription by epinephrine and norepinephrine. As a consequence, the key proteins of HCMV activation—the viral IE proteins—are synthesized. Interestingly, stimulation of HCMV activation and replication via the same cAMP-dependent intracellular signaling pathway has been demonstrated very recently for the drug pentoxifylline both *in vitro* and *in vivo* (Staak *et al.,* 1997). In contrast to TNFα-mediated stimulation of the IE enhancer/promoter, NF-κB, which acts via the 18-bp repetitive sequence motifs (Sambucetti *et al.,* 1989; Prösch *et al.,* 1995), is not or is only marginally involved in the catecholamine-induced HCMV activation.

It has been described that stress mediators may down-regulate the immune system, particularly the cell-mediated immune response, which plays a key role in the control of HCMV infection. Thus, an increase of HCMV antigenemia could simply occur due to reduced immune responsiveness. We monitored the monocytic HLA-DR and CD86 expression as well as the plasma IL-10 levels in the present study because these parameters are very sensitive indicators of stress-mediated immune depression (Woiciechowski *et al.,* 1998). In fact, the patients showed slightly increased IL-10 plasma levels (mean 7.4 pg/ml) at the time of admission but not later (<5 pg/ml). The percentage of HLA-DR and CD86 antigen-expressing $CD14^+$ monocytes did not significantly change during the follow-up period, whereas the mean fluorescence intensity was significantly decreased at days 1 and 2 by about 40–50%. Thereafter both parameters completely recovered, suggesting a moderate immunodepression, which was short-lasting and cannot explain the HCMV antigenemia peak level at day 7.

As stress is a common event, HCMV (re)activation may occur more frequently than previously thought and not only in immunosuppressed patients. Why is HCMV disease then a rare event, which is mostly restricted to immunocompromised patients? HCMV infection is controlled in particular by T-cell-mediated immunity. In fact, T-cell immunodeficiency is associated with an increased risk of developing HCMV-related disease. Using a novel flow cytometric technique we could demonstrate a very high frequency of HCMV antigen-reactive effector T-cells in the peripheral blood of healthy seropositive donors without any previous history of HCMV-related disease; up to 2% of T-cells produced IFN-γ, TNFα, or IL-2 in response to one HCMV-derived epitope within <6 h of incubation (Kern *et al.,* 1998)! The reactive T-cells ex-

press a typical effector/memory phenotype (CD11a-high, $CD28^-$). This observation gives strong evidence for a repeated contact of the immune system with HCMV antigen even in healthy persons and supports our view that HCMV activation is a common event, e.g., under stressful conditions. In nonimmunosuppressed individuals, however, HCMV antigen-expressing cells are rapidly eliminated by the specific effector T-cells. Therefore, detection of HCMV antigenemia should be a rare and very temporary event in immunocompetent humans.

It is difficult to speculate on what might be the clinical consequences of HCMV (re)activation in myocardial infarction. The fast elimination of HCMV-expressing cells within 2 weeks suggests that the T-cell immunity is not or is only temporarily diminished in these patients. This may also explain the absence of any typical HCMV disease-related symptoms. However, even if the active virus infection is rapidly controlled by T-cells, an inflammatory process may be triggered. Further studies in this direction would be interesting, particularly with respect to the discussed association between HCMV infection and the risk of restenosis after coronary atherectomy (Zhou *et al.,* 1996). Such studies may be also of interest for the understanding of the pathophysiology of several diseases for which a relation to HCMV infection is under discussion, such as cardiac allograft vasculopathy (Grattan *et al.,* 1989), atherosclerosis (Danesh *et al.,* 1997; Chiu *et al.,* 1997), and kidney transplant injury (Fishman and Rubin, 1989; Reinke *et al.,* 1994).

In summary, our data suggest that in addition to inflammatory mediators (TNFα) and some cAMP-elevating drugs (e.g., pentoxifylline) stress-induced and even therapeutically applicated catecholamines may contribute to (re)activation of HCMV infection in both immunocompromised and nonimmunocompromised patients by a direct stimulatory effect on HCMV gene expression. It further illustrates that HCMV is using very different cellular factors for its activation and replication, underscoring the excellent adaptation of the virus to its host. Commonly, a sufficient T-cell immunity controls the HCMV antigen-expressing cells in nonimmunocompromised patients but a temporary HCMV-specific T-cell-triggered inflammatory response may contribute to HCMV-associated pathophysiology.

MATERIALS AND METHODS

Patients

Ten patients (7 males, 3 females; mean age 60 years, range 46–75 years) without known infectious or inflammatory disorders and with the clinical diagnosis of acute myocardial infarction and typical ECG changes for an anterior ($n = 3$) or a posterior ($n = 7$) infarct were included in the study. The diagnosis was confirmed with serum analysis of cardiac enzymes and coronary angiograms. Four of 10 patients underwent systemic thrombolysis, 3 patients received a coronary angioplasty, and one patient needed a percutaneous transluminal coronary angioplasty after nonsuccessful thrombolysis. Three patients received catecholamine infusions because of cardiogenic shock. All but 2 patients were discharged.

The study was approved by the Institutional Review Board and informed consent was obtained from all patients.

Catecholamine determination

Catecholamine concentrations in plasma were determined using high-performance liquid chromatography as described by Ganhao *et al.* (1991).

HCMV serology

Serum IgM and IgG anti-HCMV antibodies were determined by the ELISA technique using the ETI-cytok G (Sorin Biomedica, Sallugia, Italy) and HCMV IgM-ELA test (Medac GmbH, Hamburg, Germany), respectively. Positivity was defined according to the manufacturers' criteria.

HCMV antigen test in PBMC

HCMV antigenemia was tested by immunocytology (APAAP technique) on PBMC as described previously (Fietze *et al.,* 1994) using monoclonal antibodies (mAb) recognizing HCMV immediate-early antigen-1 (Clonab CMV IE1, Clone BS 500; Biotest, Frankfurt, Germany), pp65 early matrix protein (Clonab CMV, Clone C10/C11; Biotest), or p52 early DNA binding protein (Clone CCH2; Dako, Hamburg, Germany). Isotype-matched irrelevant and anti-leucocyte common antigen mAbs (Clone: DAKO-LCA; Dako) were used as negative and positive controls, respectively. For each mAb 10,000–15,000 PBMC were studied by microscopy.

HCMV DNA PCR

For PCR, DNA was isolated from PBMC by proteinase K digestion (100 μg/ml; 1 h, 65°C) followed by phenol/chloroform extraction and ethanol precipitation as described elsewhere (Prösch *et al.,* 1992). The primers were complementary to the IE region generating a 123-bp fragment; the identity of PCR products was verified by Southern hybridization with a radiolabeled internal oligonucleotide (Olive *et al.,* 1989). Routinely 40 cycles of amplification were carried out. The lower detection threshold was at least 50 HCMV DNA copies.

Cells and virus

THP-1 cells (TIB 202) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (both certified endotoxin-free; Biochrome, Berlin, Germany) at 37°C in a 5% humidified atmosphere. The cells were

shown to be mycoplasma-free by the Mycoplasma Detection Kit (Boehringer Mannheim, Mannheim, Germany).

The β-galactosidase-expressing recombinant HCMV RC256 (constructed in the laboratory of Edward Mocarski, Stanford University) was kindly provided by J. Macziewski (NIH, Bethesda, MD). THP-1 cells were infected with an m.o.i. = 1. After adsorption was allowed to proceed for 1 h at 37°C the infected cells were cultivated as described above.

Plasmids, transfection, and CAT assay

The plasmid pRR55 contains the whole native immediate-early enhancer/promoter region of AD169 virus strain between nucleotides −671 and +52 relative to the IE-1/IE-2 transcription start site upstream of the CAT reporter gene. The plasmids p4-19PCAT, p4-18PCAT, p4-17PCAT, and p4-21PCAT carry the minimal promoter between −56 and +52 relative to the IE-1/IE-2 transcription start site and four in-tandem copies of the 19-, 18-, 17-, or 21-bp repetitive sequence motifs of the enhancer (Stamminger *et al.,* 1990). The plasmids were kindly provided by T. Stamminger (Erlangen, Germany). Plasmid p4-19/4-18PCAT, constructed in our laboratory, contains four copies of each the 19- and the 18-bp sequence motifs upstream of the minimal IE promoter and the CAT gene.

Plasmid transfection of THP-1 cells by the DEAE-dextran method and CAT assay were performed as described in detail elsewhere (Stein *et al.,* 1993).

Electrophoretic mobility shift assay

Preparation of nuclear extracts was carried out as described (Prösch *et al.,* 1995). As target DNA for EMSA we used an oligonucleotide containing the 19-bp sequence motif of the IE enhancer. Oligonucleotides were radiolabeled by filling in recessed ends with Klenow enzyme (Boehringer Mannheim) and [α-^{32}P]dCTP (Hartmann, Braunschweig, Germany). For EMSA, 9 μg of nuclear extract was incubated with 0.5 to 1.0 ng radiolabeled oligonucleotide in a 20-μl reaction volume containing the appropriate binding buffer (Prösch *et al.,* 1995; Staak *et al.,* 1997). After binding at room temperature for 20 min, the DNA–protein complexes were separated on a 5% nondenatured polyacrylamide gel.

Western blot analysis

Nuclear protein extracts (50 μg) from untreated or epinephrine/norepinephrine-treated THP-1 cells were denatured for 3 min at 100°C in Laemmli buffer, separated on a 12% SDS–polyacrylamide gel, and transferred to nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany). To avoid nonspecific binding, membranes were blocked for 12 h in TBS containing 10% inactivated neonatal calf serum, 0.2% Tween, and 0.04% NaN_3. After repeated washing, the membranes were incubated with the CREB-1 polyclonal antiserum (1:2000; PAN, Germany) for 1.5 h at room temperature, repeatedly washed, and incubated for 1 h with anti-rabbit antibody Immunopure R. Donkey (1:2000; Pierce, Rockford, IL), and again washed. To visualize the protein–antibody complexes the membrane was treated with the chemiluminiscent substrate "Supersignal Ultra" (Pierce) and exposed with X-ray film. For loading control a gel was stained with Coomassie blue.

Quantitation of viral gene expression

Epinephrine-treated and untreated THP-1 cells infected with the HCMV strain RC256 were grown for 72 h, harvested, and washed three times with PBS. After being resuspended in 500 μl PM-2 buffer the cells were subjected to five freeze–thaw cycles. After centrifugation to remove cell debris the protein concentration was measured using the Bradford reagent kit (Bio-Rad, Germany). Equal amounts of protein from cell extracts were incubated with ONPG (Sigma, Deisenhofen, Germany). The β-galactosidase activity was determined as described by MacGregor *et al.* (1991).

Statistical analysis

Data are expressed as mean ± SEM. The Friedman test and Wilcoxon test for paired samples were used for comparison of dependent values in follow-up. The Pearson coefficient with two-tailed significance levels was taken for correlation analyses. Differences were considered significant at $P < 0.05$.

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