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Reduced Expression of Gamma Interferon in Serum and Marked Lymphoid Depletion Induced by *Porphyromonas gingivalis* Increase Murine Morbidity and Mortality due to Cytomegalovirus Infection

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***Porphyromonas gingivalis*, a gram-negative anaerobe, is a major etiological agent of severe forms of periodontal disease. Although periodontal disease is considered a localized disease, accumulating evidence indicates that it may lead to a predisposition to a decline in immunocompetence. Human cytomegalovirus (CMV) commonly infects all human populations without producing significant clinical symptoms. Immunocompromised patients usually develop a primary or reactivated CMV infection, which is associated with high rates of morbidity and mortality. The aim of this study was to determine whether *P. gingivalis* increases animal susceptibility to CMV infection. Mice were inoculated with CMV and infected locally with *P. gingivalis* 3 days after the virus inoculation. Mortality rates were monitored, and traces of viral DNA and bacterial infection were detected systemically by using real-time PCR. Local and systemic cytokine secretion was measured, and histological sections were used to assess the pathological state of infected organs. *P. gingivalis*- and CMV-coinfected mice showed dramatically higher mortality rates than mice infected with *P. gingivalis* or CMV only. Although the organs of coinfected mice exhibited decreased viral titers, distinct necrosis and tissue damage were more evident in the livers and spleens of these mice than in those of mice infected with CMV only. Furthermore, systemic gamma interferon levels were decreased in coinfected mice, and marked lymphoid depletion was observed in their necrotic organs. In parallel control *Escherichia coli*-CMV coinfection experiments, the mortality and pathological results were the same as those found in mice infected with CMV only. Our results suggest a specific influence of *P. gingivalis* on the mouse immune response, causing increased susceptibility to CMV infection.**

Human cytomegalovirus (HCMV), a member of the herpesvirus family, commonly infects all human populations without producing significant clinical symptoms. Following a primary infection, the host develops mild subclinical illness, after which the virus enters a latent phase that usually persists for life. In the prenatal period, a reactivated (or primary) HCMV infection of the pregnant woman may cause severe congenital defects in the newborn even though the mother has no apparent clinical symptoms (10). HCMV disease becomes a serious hazard to the host when the latent virus is reactivated as a result of changes in the immunocompetence of the host.

In immunocompromised hosts, reactivation of the latent virus leads to single-organ or multiorgan HCMV disease associated with high rates of morbidity and mortality (33). It is one of the most common infections among AIDS patients, and patients undergoing bone marrow or solid organ transplantation tend to develop a primary or reactivated HCMV infection. HCMV is a formidable barrier to the success of bone marrow transplantation and is considered a major cause of morbidity and mortality in these patients (39). The clinical characteristics of HCMV infection are extremely variable, but interstitial pneumonia is the most serious form. Until recently, HCMV interstitial pneumonitis occurred in 20% of transplant patients and resulted in over 80% mortality. Therefore, it is clear that the immune system plays a pivotal role in the control of HCMV. As long as the immune system can provide adequate protection, HCMV infection remains latent and asymptomatic in seropositive individuals. Any change in the balance of the immune response may threaten the lives of immunocompromised patients.

Porphyromonas gingivalis, a gram-negative anaerobe, is a major etiological agent in the initiation and progression of periodontal disease, mainly in the severe forms of the disease. These severe forms of periodontal disease can be found in 5 to 15% of the population and are characterized by a severe inflammatory response followed by a loss of bone and tooth attachment (3). Periodontal disease has also been reported to contribute to the development of several systemic conditions, such as coronary heart disease, low birth weight, and diabetes (23). Moreover, the severe inflammation of the periodontal tissues caused by *P. gingivalis* has been reported to change the immune balance of the host and may cause a reduction in host systemic immunocompetence (20, 26). Recently, an impaired immune response caused by exposure of mice to *P. gingivalis* was described; bacterial antigens induced generalized T-cell dysfunction through down-regulation of the T-cell receptor ζ chain (5).

Based on the influence of *P. gingivalis* on the host immune

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response, we theorized that immunosuppressed individuals who have a concomitant periodontal infection are at risk for an additional decline in their immune defenses. This notion suggests that immunocompromised patients coinfected by HCMV and *P. gingivalis* may develop a more severe and rapid lethal disease. We used a mouse model resembling the clinical state described above to test our hypothesis that infection with *P. gingivalis* during CMV infection in immunocompromised mice may increase the susceptibility of the animals to the viral infection. CMV infection in the mice was created with murine CMV (MCMV), which is a natural pathogen of mice and which resembles HCMV in physical structure and pathogenesis. Like the pathogenesis of HCMV infection, the pathogenesis of MCMV infection is characterized by the development of acute and latent phases of infection. Primary infection is associated with virus replication in various types of tissues at multiple organ sites. The extent of virus production and the development of MCMV disease, however, depend largely on the immune status of the host (18).

To create a localized infection with *P. gingivalis* in mice, we used the in vivo subcutaneous chamber model (11). In addition to the encapsulated environment that resembles a localized infection, this model offers an efficient study tool. In this study, we examined some specific mechanisms involved in the bacterial infection, the influence of the bacterial infection on the progression of the viral infection, and their mutual effects on the host immune response. Unlike most previous studies that dealt with viral and bacterial coinfection and focused on virus-induced immunomodulation, this coinfection study is directed mainly at elucidating the bacterial influence on the host immune response. Herpesvirus-*P. gingivalis* coinfection was previously studied (34) and found to be correlated with periodontal disease and its components. Earlier studies concentrated on the contribution of viral infection to localized periodontal disease. In this study, we focused on the bacterial influence on the host immune response and its contribution to the progress of the viral disease. The results of this study may help to define this coinfection as another risk factor for immunocompromised patients, such as transplant recipients and AIDS patients, implying the need for new limitations and strict prophylactic periodontal treatments that will improve patient care.

MATERIALS AND METHODS

Animal and study protocol. BALB/c mice were bred at the Hebrew University specific-pathogen-free facility. Animals were used in accordance with protocols approved by the Hebrew University-Hadassah Medical School Institutional Animal Care and Use Committee. Experiments were performed with four groups of six to eight infected mice. In addition to the experimental groups of *P. gingivalis*- and MCMV-coinfected mice, groups of mice infected with *P. gingivalis* only or MCMV only and *Escherichia coli*- and MCMV-coinfected mice served as controls. Chambers constructed from coils of titanium wire were implanted subcutaneously in healthy 5- to 6-week-old BALB/c mice through a middorsal incision under ketamine anesthesia. After a minimal 2-week recovery period, the mice were immunosupressed with 150 mg of cyclophosphamide (CY)/kg of body weight and injected with 10^3 PFU of MCMV. CY and MCMV were given intraperitonealy on the same day. At 3 days after viral infection, mice received an intrachamber injection of *P. gingivalis* (10^9 CFU in a 100-μl volume). Control mice were subjected to the same protocol but were injected with phosphate-buffered saline (PBS) or *E. coli* (w3110; 10^{10} CFU/ml). During the experiments, the mice were kept in specific-pathogen-free conditions and observed daily. In experiments where mortality was not observed, two to four animals from each group were sacrificed at various time points, and organs, sera, and chamber exudates were pooled for microbiological, immunological, and histological evaluations. All data from three independent experiments were collected and analyzed.

Bacterial preparation. *P. gingivalis* ATCC 33277 was grown on blood agar plates in an anaerobic chamber with 85% N_2–5% H_2–10% CO_2. After incubation at 37°C for 2 to 3 days, the bacterial cells were inoculated into peptone-yeast extract for 1 week of incubation under the same conditions. The bacteria were washed three times with PBS. By use of a spectrophotometer, the bacterial concentration was standardized to an optical density at 650 nm of 0.1, which corresponds to 10^{10} CFU/ml.

Virus and virus titration. MCMV strain Smith was obtained from the American Type Culture Collection, Manassas, Va. A preparation of a highly virulent virus stock from MCMV-infected salivary glands was prepared as described previously (17). Vials containing 10^7 PFU/ml were stored at −70°C in 10% dimethyl sulfoxide until used. A semiquantitative plaque assay on primary cultures of mouse embryo fibroblasts (28) was used to titrate infectious MCMV.

Detection of *P. gingivalis* in the host. The presence of *P. gingivalis* during the course of infection was assessed with serum and chamber exudate samples from all groups. Samples, taken at 8, 24, 32, 48, and 72 h after bacterial inoculation, were cultured on blood agar plates by incubation at 37°C in an anaerobic chamber and checked 7 days later for the presence of black-pigmented colonies (6). DNA was extracted from cultures, and the *fimA* gene of *P. gingivalis* was detected by real-time PCR to confirm the presence of the specific bacteria.

Extraction of DNA. PCR for detection of viral DNA was carried out with salivary gland, liver, spleen, and lung samples. These tissues were dissected and homogenized with a Polytron homogenizer (Kinematika, Lucerne, Switzerland), and DNA was extracted by use of EZ-DNA reagent (Beit-Haemek, Kibutz Beit Haemek, Israel).

Detection and quantification of MCMV and *P. gingivalis* DNAs by real-time PCR. A 500-ng DNA sample was used in a 20-μl PCR mixture. Amplification was done by use of a LightCycler (Roche Molecular Biochemicals) with a standard PCR mixture containing 0.5 μM each primer and SYBR Green I reaction mix (Roche). For MCMV DNA quantification, reactions were cycled 45 times with denaturation at 95°C for 10 s, annealing at 70°C for 25 s, and extension at 72°C for 25 s. Fluorescence was acquired at the end of the extension phase. The melting curves were obtained at the end of amplification by cooling the sample at 20°C/s to 65°C and then increasing the temperature to 95°C at 0.1°C/s. Fluorescence was acquired every 0.1°C. Primers for the MCMV immediate-early gene were used as described by Balthesen et al. (2), and plasmid pIE111, which contains the immediate-early gene, was used as a standard.

Reaction conditions for *P. gingivalis fimA* detection were 45 cycles of denaturation at 95°C for 10 s, annealing at 59°C for 20 s, and extension at 72°C for 20 s. Fluorescence was acquired at the end of the extension phase. The melting curves were obtained at the end of amplification by cooling the sample at 20°C/s to 55°C and then increasing the temperature to 95°C at 0.1°C/s. Fluorescence was acquired every 0.1°C. Primers for the *P. gingivalis fimA* gene were used as described by Watanabe and Frommel (37).

ELISA of cytokines. To assess the characteristic host immune response to the viral and bacterial infections, inflammatory (tumor necrosis factor alpha [TNF-α] and gamma interferon [IFN-γ]) and humoral (interleukin-10 [IL-10]) cytokine levels were measured. The secreted forms of these representative cytokines were quantified with serum and chamber exudate samples. Analysis was done by use of a two-site enzyme-linked immunosorbent assay (ELISA) based on commercially available antibody pairs as described previously (31). Briefly, 96-well ELISA plates were coated with an anticytokine monoclonal antibody in a coating buffer (carbonate-bicarbonate buffer [pH 9.6]), followed by overnight incubation at 4°C. The wells were blocked for 3 h at room temperature with 3% bovine serum albumin in the coating buffer. Samples then were added and incubated overnight at 4°C. A second, cytokine-specific, biotin-conjugated monoclonal antibody was used as the detection antibody, and the reaction was developed with strepavidin-horseradish peroxidase conjugate and nitroblue tetrazolium as the substrate. The reaction was stopped by the addition of 2 M sulfuric acid, and the optical density at 450 nm was read by use of a Vmax microplate reader (Molecular Devices, Palo Alto, Calif.).

Histological procedures. Livers, lungs, spleens, and salivary glands were dissected and fixed in 4% buffered formaldehyde (Bio Lab Ltd., Jerusalem, Israel). After fixation, samples were dehydrated in a series of graded ethanol solutions and embedded in paraffin according to standard techniques. Sections were cut at 5 mm and stained with hematoxylin and eosin for pathological evaluation.

Statistical methods. The Kaplan-Meier survival analysis model was used to assess the survival of mice in the different treatment groups, and the log-rank test was applied to compare survival curves between groups.

Analysis of variance with repeated measures was applied to test for differences

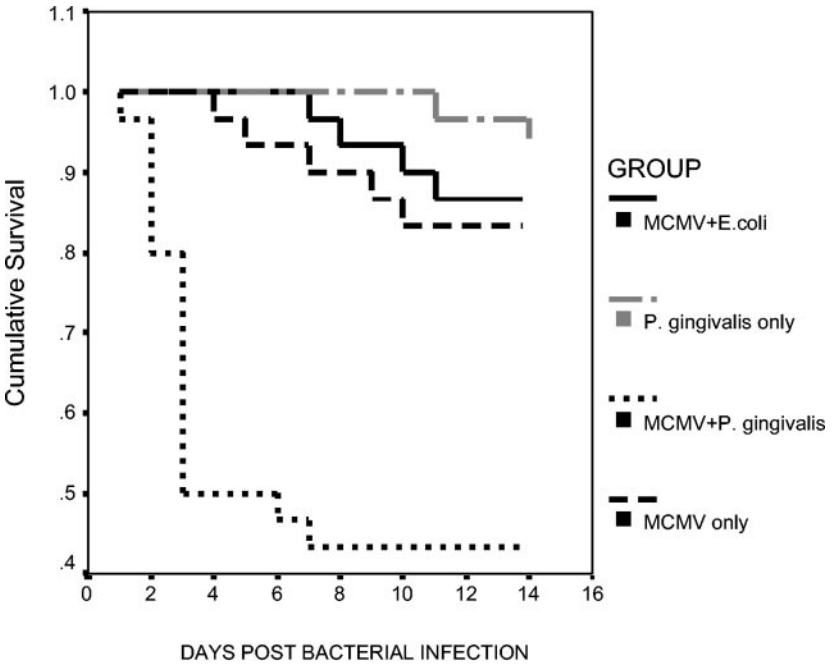

FIG. 1. Survival functions for all infected mice. At 3 days after MCMV infection, six to eight mice in each experimental group were injected in the implanted chambers with *P. gingivalis*, *E. coli*, or PBS. Morbidity and mortality rates were monitored daily and compared for all of the groups up to day 14 after bacterial infection. These data summarize the results of four independent experiments with six to eight mice in each group in every experiment. The Kaplan-Meier survival analysis model was used to assess the survival of mice in each group (total $n = 30$). The P value, determined by the log rank test, was <0.001.

between treatment groups, changes in measurement levels over time, and the interaction between them for continuous variables.

The Kruskal-Wallis and Mann-Whitney nonparametric tests were used to compare treatment groups for three and two groups, respectively.

All tests applied were two-tailed, and a P value of 5% or less was considered statistically significant.

RESULTS

Increased mortality rates in *P. gingivalis*- and MCMV-coinfected mice. To examine whether infection with *P. gingivalis* during CMV infection increases the susceptibility of animals to the viral infection, we initially compared the mortality rates of the different study groups. Morbidity and mortality rates were monitored and compared for all of the groups up to 14 days after viral inoculation. At 2 to 3 days after bacterial inoculation, *P. gingivalis*- and MCMV-coinfected mice showed severe illness that was manifested by slow apathetic behavior and scruffy fur with a focal area of gray-green discoloration on the skin surrounding the chamber. Mice infected with *E. coli* and MCMV or with MCMV only showed mild illness, and the progression of the disease was much slower. The death of *P. gingivalis*- and MCMV-coinfected mice occurred from days 2 to 4 after bacterial inoculation, reaching mortality rates of 55% (Fig. 1). For mice infected with *E. coli* and MCMV or with MCMV only, mortality rates reached only 18 and 24%, respectively, with death occurring from days 6 to 11. Mortality rates for mice infected with *P. gingivalis* only were merely 6% (Fig. 1). For an additional control group infected with MCMV and heat-killed *P. gingivalis*, the results were similar to those for the group infected with MCMV only (20% mortality rate).

MCMV-induced suppression of local cytokine secretion in response to *P. gingivalis* infection. Samples from chamber exudates were taken from all infected mice at various time points. Levels of TNF-α, IFN-γ, and IL-10 in the chambers were measured by an ELISA and compared for all of the groups at all of the time points. To determine a baseline, a control group of mice inoculated with CY only was added. *P. gingivalis*- and MCMV-coinfected mice showed lower levels of TNF-α, IFN-γ, and IL-10 expression in the chambers than did mice infected with *P. gingivalis* only (Fig. 2). Mice infected with MCMV only showed no significant cytokine expression, indicating that all immune changes that were measured in the chambers were induced by the presence of *P. gingivalis*. In all *P. gingivalis*-infected groups, the patterns of expression of all of the cytokines were similar, i.e., reaching a peak 24 h after infection (Fig. 2).

Marked tissue damage around the chambers in MCMV- and *P. gingivalis*-coinfected mice. At 3 days after bacterial inoculation, skin and muscle in the vicinity of the infected chambers were taken for histological evaluation. Necrotic debris with evidence of bacteria and a leukocyte infiltrate was found in deep dermal and pannicular areas as well as in muscle fibers around the chambers in *P. gingivalis*- and MCMV-coinfected mice (Fig. 3, panels B). No such tissue damage was found around the chambers in control mice (Fig. 3, panels A).

Qualitative detection of live *P. gingivalis* in blood and chambers. To ensure the presence of living bacteria throughout the experiment and to evaluate their invasive abilities, blood and chamber samples were collected from all groups of mice. Samples were taken at 8, 24, 32, 48, and 72 h after bacterial

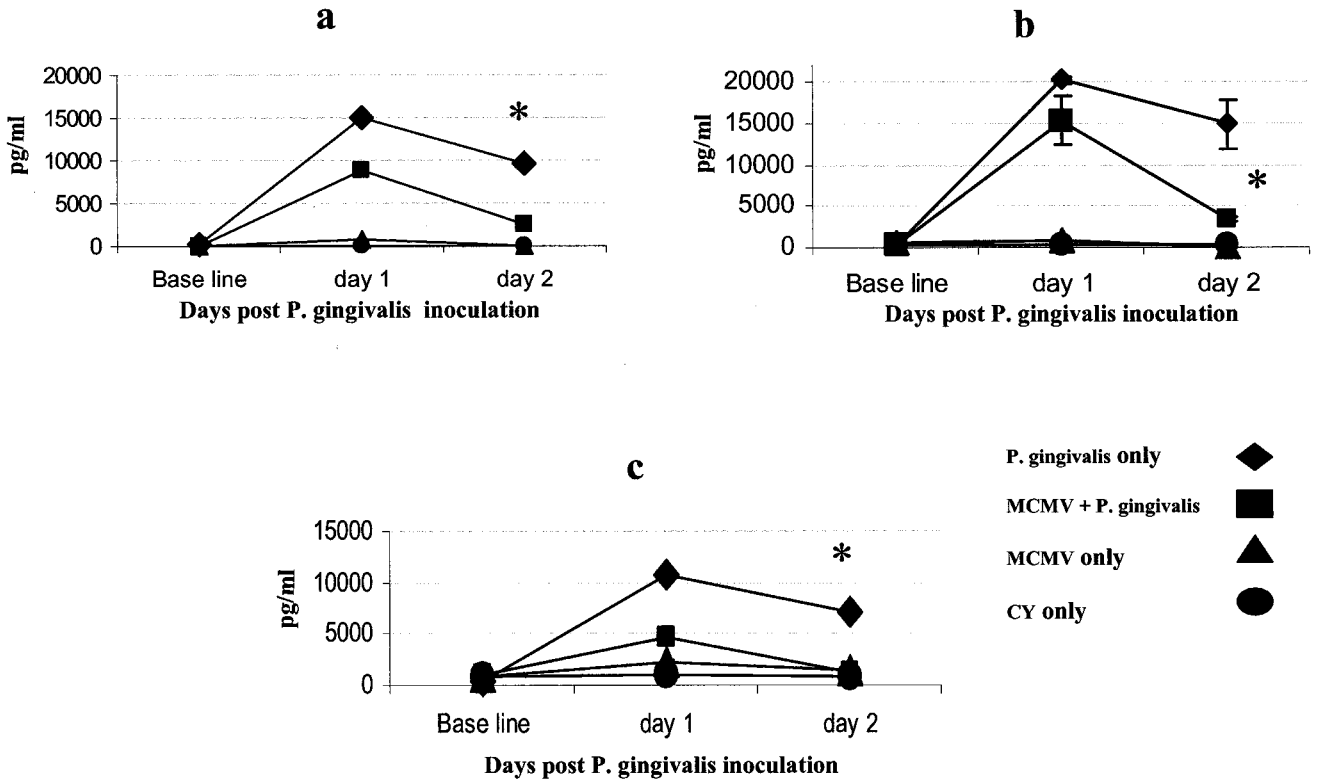

FIG. 2. MCMV-induced changes in local immune responses. Levels of TNF-α (a), IL-10 (b), and IFN-γ (c) secretion in the chambers were measured by an ELISA at 24 and 48 h after bacterial inoculation. These data summarize the results of three independent experiments with four mice in each group in every experiment (total $n = 12$). Error bars indicate standard deviations. An asterisk indicates a P value of <0.001.

inoculation. Samples were cultured on blood agar plates, and *P. gingivalis* colonies were counted. At all times in both *P. gingivalis*-infected groups, live *P. gingivalis* was detected in chambers, and from 32 h after bacterial inoculation, live *P. gingivalis* was detected in the blood circulation as well. To confirm the presence of the specific bacteria, DNA was extracted from cultures, and the *fimA* gene of *P. gingivalis* was detected by nonquantitative PCR.

Quantification of viral DNA in infected mice. To plot the tendencies for viral propagation, spleens and livers were har-

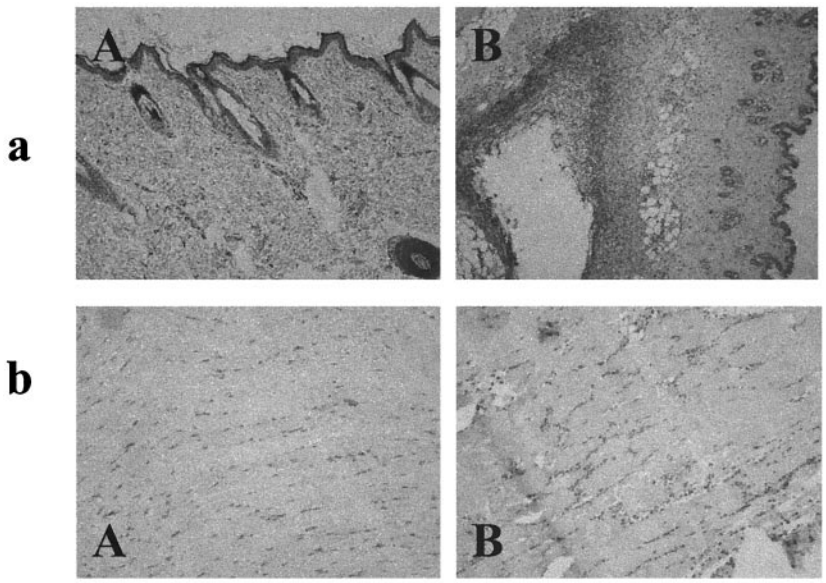

FIG. 3. Histological changes around the chambers. At 3 days after bacterial inoculation, skin (a) and muscle (b) in the vicinity of the infected chambers were taken from all groups for histological evaluation. (A) Control mice. (B) MCMV- and *P. gingivalis*-coinfected mice.

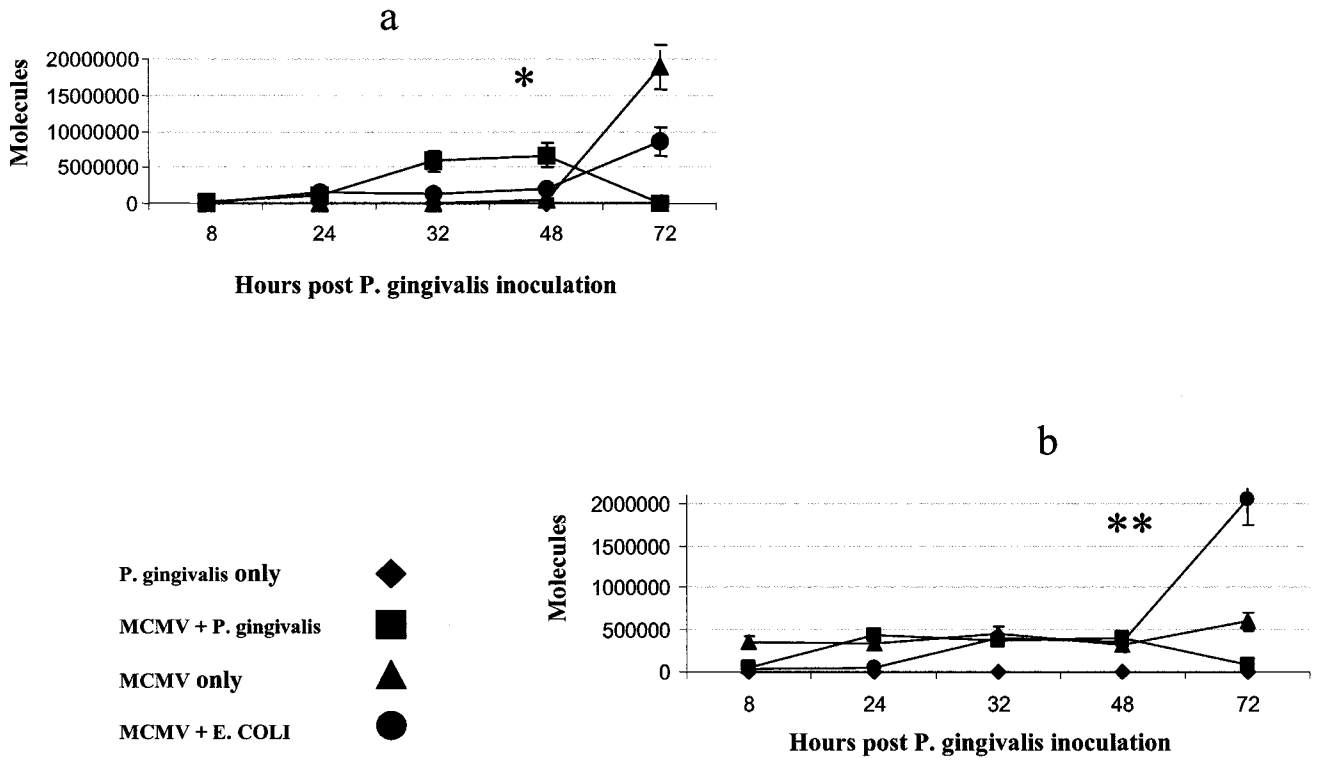

FIG. 4. Amounts of viral DNA in infected mice. Spleens (a) and livers (b) from two mice in each group were harvested at different time points for DNA extraction. Viral DNA was quantified by real-time PCR, and viral propagation tendencies were estimated and compared for all of the groups. These data summarize the results of three independent experiments (total $n = 6$). Error bars indicate standard deviations. A single asterisk indicates a P value of <0.025; a double asterisk indicates a P value of <0.027.

vested from infected mice at various time points for DNA extraction and quantification. Viral DNA was quantified by real-time PCR, and viral loads were compared for all of the groups. Spleens (Fig. 4a) and livers (Fig. 4b) from *P. gingivalis*- and MCMV-coinfected mice showed decreasing amounts of viral DNA after 48 h. In contrast, spleens and livers from mice infected with MCMV only or from mice coinfected with *E. coli* and MCMV showed increasing amounts of viral DNA (Fig. 4). To confirm that these results reflected changes in the amounts of living virus and not just DNA particles, live virus was quantified by a plaque assay 5 days after viral infection (72 h). *P. gingivalis*- and MCMV-coinfected mice exhibited 10-fold less PFU than did mice infected with MCMV only.

Decreased expression of IFN-γ in the serum of *P. gingivalis*- and MCMV-infected mice. At various time points, blood samples were taken from all infected mice. The levels of IFN-γ in the serum were measured by an ELISA and compared for all of the groups. At all time points, *P. gingivalis*- and MCMV-infected mice showed lower levels of IFN-γ expression than did mice infected with MCMV only. At 72 h after bacterial inoculation (6 days after viral inoculation), *P. gingivalis*- and MCMV-coinfected mice showed sixfold lower IFN-γ levels than did mice infected with MCMV only (Fig. 5).

Decreased cellular immune response in organs of *P. gingivalis*- and MCMV-coinfected mice. Histological sections of livers and spleens taken from infected mice at days 2 and 3 after

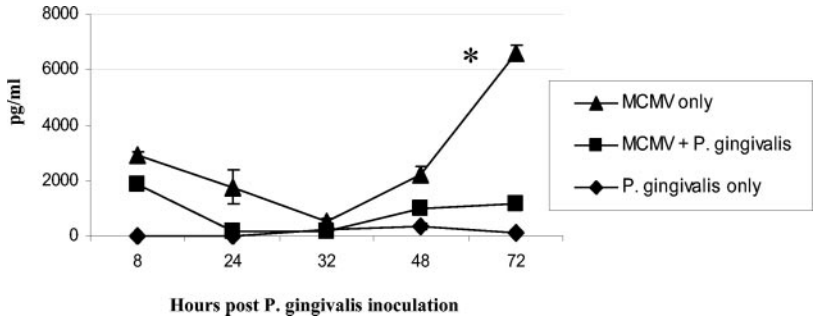

FIG. 5. Comparison of serum IFN-γ levels. At different time points, blood was pooled from two infected mice in each group for ELISA measurements of serum IFN-γ levels. These data summarize the results of three independent experiments (total $n = 6$). Error bars indicate standard deviations. An asterisk indicates a P value of <0.001.

bacterial inoculation were analyzed for pathological damage. Livers from *P. gingivalis*- and MCMV-coinfected mice showed multifocal areas of necrosis with a minimal leukocyte infiltrate. Occasional aggregations of macrophages were evident in perivascular areas and rarely in areas of necrosis. These mice exhibited a decreased immune response compared to that of control mice and compared to what is expected when this kind of profound tissue damage exists. Livers from mice infected with MCMV only or from *E. coli*- and MCMV-coinfected mice showed random multifocal areas of necrosis with aggregations of macrophages and lymphocytes infiltrating the areas of necrosis. Livers from mice infected with *P. gingivalis* only showed no significant pathological findings (Fig. 6a).

Spleens from all MCMV-infected mice demonstrated evidence of necrosis at the marginal areas of germinal centers and within the interstitium and mild lymphoid depletion. Spleens from *P. gingivalis*- and MCMV-coinfected mice showed the most severe tissue damage, with abundant pyknosis within lymphoid follicles, karyorrhexis, and focal necrosis. These spleens also showed marked lymphoid depletion, with only small aggregations of small lymphocytes remaining. Again, as with the liver histological findings, *P. gingivalis*- and MCMV-coinfected mice exhibited a minimal immune response relative to the severe tissue damage. Germinal centers in spleens of mice infected with *P. gingivalis* only appeared normal, with only numerous megakaryocytes and neutrophils being evident within the interstitium (Fig. 6b). Other organs, such as the lungs, heart, kidneys, adrenal glands, salivary glands, urinary bladder, stomach, and intestines, showed no significant pathological findings.

DISCUSSION

Immunocompromised patients infected with HCMV are more susceptible to opportunistic infections that can rapidly become lethal. HCMV disease is one of the most common infections among AIDS patients, reaching a prevalence of 100% in most human immunodeficiency virus-infected groups of patients (4). About 50% of transplant patients excrete HCMV in body secretions at some stage after organ transplantation, and a symptomatic disease occurs in 40 to 60% of patients who experience a primary infection (32).

Periodontal disease, a localized disease found in 30 to 35% of the population in the United States, is characterized by loss of bone and tooth attachment and the creation of deep periodontal pockets that can extend to the entire length of the tooth roots. Several species of bacteria are associated with the disease, including *P. gingivalis*, which is considered a major periodontal pathogen (3). Although *P. gingivalis* is considered to generate a localized disease, evidence is accumulating that this pathogen may have systemic effects. Based on previous studies that showed that *P. gingivalis* may have a systemic influence on the host immune response (20), this study was designed to evaluate whether coinfection by *P. gingivalis* and MCMV in immunocompromised mice might result in more severe and rapid development of a lethal disease.

In this study, mice were inoculated with MCMV and treated with CY. On day 3 after viral inoculation, mice were also infected locally with *P. gingivalis*. The 3-day interval between the two inoculations was based on previous studies (1, 22) and is needed because of the different kinetics of progression of viral and bacterial infections. To create a localized infection with *P. gingivalis* in mice, we used the in vivo subcutaneous chamber model (11). Although periodontal tissue and the subcutaneous tissue around the chamber differ in several aspects, in both cases the localized infection can serve as a chronic reservoir for bacteremia induced by *P. gingivalis*. In fact, chronic periodontal disease infection is capable of causing an ulcerated lesion at the dental-epithelial junction that is estimated to range from 8 to 20 cm^2 (15), easily allowing bacterial invasion of the systemic circulation.

The most convincing and easily monitored indication for any influence of *P. gingivalis* on a CMV-infected host is the mortality rate. The significant high mortality rates found in *P. gingivalis*- and MCMV-infected mice compared to *E. coli*- and MCMV-infected mice clearly demonstrated that the presence of *P. gingivalis* affects the host immune response to CMV infection and warrant further examination.

Several experiments were conducted to better understand the role of *P. gingivalis* in this coinfection. Viral DNA and traces of bacterial infection were monitored systemically in order to follow the progression of infections with both pathogens. Local and systemic cytokine secretions were measured to evaluate the immune response, and histological sections were used to assess the pathological status of the infected organs during the course of infection.

The first evidence of a severe effect caused by the presence of the two pathogens was seen in the histological evaluation of the tissues surrounding the chamber. Pathological damage, found only in *P. gingivalis*- and MCMV-coinfected mice, suggested that MCMV infection might alter the bacterium-host interaction. HCMV-induced immunosuppression was well documented in previous studies (25, 27, 29). Regarding periodontal disease, accumulating data collected by various investigators support the concept that HCMV contributes to severe types of periodontal disease by altering the immune control of local microorganisms (7, 24). HCMV coinfection of the periodontium can be important in multistage pathogenesis of periodontitis involving periodontopathic bacteria, activation of the virus, and host immune responses. The decreased local immune response to *P. gingivalis*, as expressed by the lower levels of cytokine secretion in the chambers in *P. gingivalis*- and MCMV-coinfected mice, correlates with the studies mentioned above.

Live bacteria were found in the chambers in all *P. gingivalis*-infected groups at all time points, indicating that the pathogen was still viable during the progression of the disease. To evaluate whether the presence of *P. gingivalis* was limited to the chambers, we qualitatively searched, by cultivation, for the existence of living bacteria in blood samples from all groups of mice. Although there was evidence of *P. gingivalis* in the serum, we did not further examine virus-induced changes in the immune response away from the chamber, since the main aim of this study was to concentrate on *P. gingivalis* and its influence on the systemic immune response against MCMV infection.

Plaque assay and PCR quantification of viral DNA particles in all groups were done to estimate the influence of *P. gingivalis* on viral propagation. The lower viral titers found by both detection methods at 72 h after bacterial infection in the livers and spleens of *P. gingivalis*- and MCMV-coinfected mice are not completely understood. These results are further puzzling when taken together with the higher levels of pathological

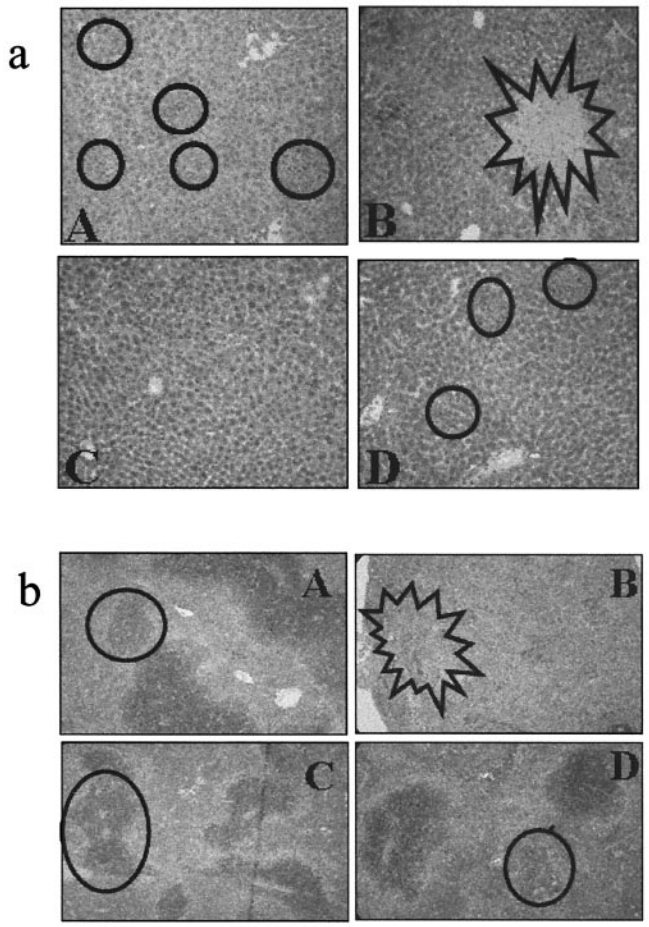

FIG. 6. Pathological evaluation of infected livers and spleens. At 2 and 3 days after bacterial inoculation, mice were sacrificed and samples of infected livers (a) and spleens (b) were taken for histological evaluation. Although *P. gingivalis*-and MCMV-coinfected mice (B) showed marked necrosis (sunburst outline) in livers and spleens, their lymphatic immune responses (circle outline) were poor compared to those of mice infected with MCMV only (A) or MCMV and *E. coli* (D). (C) Infected with *P. gingivalis* only.

damage and lower levels of serum IFN-γ found in these same coinfected mice. IFN-γ is an important mediator of MCMV clearance (8); lower levels of this cytokine together with marked tissue damage would instead imply high viral titers. However, the number of CMV-infected cells is not always a reflection of clinical disease severity (32). In humans, large numbers of CMV-infected cells may be present in tissues of asymptomatic patients, whereas small numbers of CMV-infected cells may be present in those with severe or fatal disease (35). The fact that these lower viral titers occurred only in the presence of *P. gingivalis* and not in the other control groups suggests a *P. gingivalis*-specific influence on viral activity. Such influence might be explained by a direct effect, such as secretion of an antiviral substance by *P. gingivalis*, or by an indirect effect, such as tissue damage that depletes the virus from propagation sources and thus lowers its titers.

The influence of bacteria on the cellular host immune response was estimated by histological analysis of the infected organs. *P. gingivalis*- and MCMV-coinfected mice demonstrated a minimal lymphocytic infiltrate in the liver and marked lymphoid depletion in the spleen. These findings suggest that the host immune response in this group was somehow suppressed. Since this same group of mice also showed lower levels of serum IFN-γ than the control groups, we suggest that some type of systemic immunosuppression is specifically induced by the presence of *P. gingivalis* infection. Whether the immunosuppression induced by *P. gingivalis* is mediated through the decrease in serum IFN-γ levels is still to be determined. However, IFN-γ is known to be an antiviral cytokine and is an important key mediator in the protection of the host against CMV (8). Previous studies showed the role of IFN-γ in the T-cell-mediated control of CMV infection (12, 13, 14).

The pathological damage in coinfected mice was focused mainly in the liver and the spleen. Other organs, such as the lungs, heart, kidneys, and salivary glands, showed no significant pathological findings. A preliminary study that included biochemical blood tests for the analysis of liver enzymatic activity showed extremely high levels of alanine aminotransferase (ALT) in the serum followed by very low levels only in the *P. gingivalis*- and MCMV-coinfected group (data not shown). Extremely high and low levels of ALT are usually induced by liver necrosis, meaning that immediately after hepatocyte death, this enzyme stored in vacuoles is dispensed into the bloodstream and disappears after a short time. The distinct necrosis observed in histological sections of the livers of *P. gingivalis*- and MCMV-coinfected mice, which correlates with the high levels of ALT observed in the serum, suggests that liver failure caused by the viral infection can be considered the cause of death.

P. gingivalis- and MCMV-coinfected mice displayed severe illness and increased mortality, which occurred mostly on days 2 to 3 after bacterial inoculation. Mice infected with *E. coli* and MCMV or MCMV only showed mild illness, and the progression of the disease was much slower. The control group infected with heat-killed *P. gingivalis* and MCMV also showed mild disease, indicating that the bacterial vitality in the host is crucial to its potential influence. These results correlate with the fact that many *P. gingivalis* virulence factors, including those related to immunosuppression, are known to be controlled and regulated by the bacteria according to environmental changes (21), obviously depending on bacterial vitality (further discussed below). In addition, the heat-killed *P. gingivalis* control group and the *E. coli* control group, which together represent a general model for gram-negative bacteria, emphasize the uniqueness of *P. gingivalis* virulence and its exclusive interactions with the host. Studies on *P. gingivalis* interactions with host immune mechanisms have shown that although lipopolysaccharide has a significant role in the initial arrest of host defense, *P. gingivalis* possesses and uses a wide variety of other virulence factors. Proteolytic enzymes of *P. gingivalis* can degrade complement components, cytokines, and antibodies (9, 16, 36). *P. gingivalis* is able to affect polymorphonuclear cell function (38); e.g., low-molecular-weight fatty acids produced by *P. gingivalis* are able to inhibit polymorphonuclear cell chemotaxis (30).

Metabolic by-products, such as butyric acid, produced by *P. gingivalis* can inhibit proliferation and induce the death of lymphocytes (19). Adhesion molecules, including the *P. gingivalis* unique FimA molecule, are used by the bacteria to manipulate intracellular signal transduction pathways (21). The ability of vital *P. gingivalis* to penetrate tissues, to internalize

into cells, and to regulate its own gene expression according to environmental conditions may assist the organism in avoiding host immune cells and extend its survival in the host (21).

In conclusion, although HCMV is known to have an immunosuppressive effect, we show that the systemic influence on the host immune response, resulting in increased mortality rates, was caused by the presence of *P. gingivalis* infection. We believe that the virus-induced suppression of the local immune response against *P. gingivalis* enables the bacteria in the chambers and later in the serum to enhance the pathological influence of the virus on the host, finally leading to severe and rapid illness. In other words, a synergistic effect resulting from the presence of the two pathogens, where CMV assists *P. gingivalis* in promoting and elevating the influence of the virus on the host, may exist. These findings suggest that the presence of *P. gingivalis* in periodontal tissues can be considered an important risk factor in patients with any kind of immunosuppression. In situations where the host is susceptible to lethal opportunistic infections, the presence of chronic periodontal disease caused by *P. gingivalis* can actually serve as a catalyzing factor in worsening the disease outcome.

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