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Monocyte Chemoattractant Protein 1 and Interleukin-8 Production in Mononuclear Cells Stimulated by Oral Microorganisms

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Chemokines are a family of low-molecular-weight proinflammatory cytokines that stimulate recruitment of leukocytes. The chemokines interleukin-8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1) are relatively specific chemoattractants for neutrophils and monocytes, respectively. Chemokine expression contributes to the presence of different leukocyte populations observed in normal and pathologic states. In the present studies, peripheral blood mononuclear cells (PBMC) were stimulated by microbes (*Candida albicans*, *Streptococcus mutans*, *Porphyromonas gingivalis*, and *Actinobacillus actinomycetemcomitans*) selected based upon their importance as oral pathogens. IL-8 and MCP-1 gene expression and protein release were determined by Northern blot (RNA blot) analysis and enzyme-linked immunosorbent assay. *C. albicans*, *P. gingivalis*, and *A. actinomycetemcomitans* induced high levels of production of both MCP-1 and IL-8. *S. mutans* was a strong inducer of MCP-1, but it did not stimulate significant production of IL-8. *C. albicans*, *S. mutans*, and *A. actinomycetemcomitans* were 500 to 5,000 times more potent than *P. gingivalis* in terms of MCP-1 production. In general, the microbe-to-PBMC ratios required for maximum gene expression of MCP-1 were lower than those for IL-8. However, for actual protein release of MCP-1 versus IL-8, differences in the effects of various microbe concentrations were observed only for *A. actinomycetemcomitans*. These results demonstrate that different oral pathogens induce specific dose-dependent patterns of chemokine gene expression and release. Such patterns may help explain the immunopathology of oral infections, particularly with regard to inflammatory leukocyte recruitment.

Commonly seen oral infections include periodontitis, pulpitis, lesions of endodontic origin, and candidiasis. The gram-negative bacteria *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* are putative periodontal pathogens. *P. gingivalis* may also contribute to the periradicular pathology seen in lesions of endodontic origin (4, 6, 19). *A. actinomycetemcomitans* can affect immune responses by production of potent virulence factors including endotoxin, leukotoxin (21, 28), and a chemotaxis inhibiting factor (22). *Streptococcus mutans*, a gram-positive bacterium, is a major component of dental plaque and may contribute to periapical inflammation (12, 15). *Candida albicans* is the usual etiologic agent of oral mucocutaneous candidiasis and is frequently seen in immunodeficient individuals, particularly those with AIDS.

The invasion of host tissue by microbes or their products frequently induces a wide variety of immunopathologic reactions. Polymorphonuclear leukocytes (PMNs) and mononuclear phagocytes are key components of host defenses against invading microbes. PMNs tend to predominate in acute infections, whereas a mononuclear infiltrate is more often seen in chronic infections. The nature of the leukocyte infiltrate affects the progress of the disease. Therefore, factors that regulate the recruitment of specific leukocytes represent an important component of the host response.

Chemokines are a recently described family of chemotactic cytokines. Unlike the classic leukocyte chemoattractants, they exhibit a relatively high degree of specificity. Interleukin-8 (IL-8) is the most thoroughly characterized neutrophil-stimulating chemokine. IL-8 displays a wide range of biologic effects, including chemotaxis and activation of neutrophils. High levels of IL-8 are seen after septic shock or systemic administration of endotoxin (10). Monocyte chemoattractant protein 1 (MCP-1) stimulates chemotaxis of monocytes but not neutrophils.

Mononuclear phagocytes are thought to be the predominant cellular sources of both IL-8 and MCP-1 (9, 27). Blood monocytes secrete large amounts of these chemokines when appropriately stimulated. Peripheral blood lymphocytes are not known to secrete MCP-1 (1), although they may produce IL-8 in response to certain stimuli (2). However, MCP-1 and IL-8 can also be produced by nonimmune cells such as fibroblasts, keratinocytes, and endothelial cells, in response to both endogenous and exogenous stimuli (3). Expression of MCP-1 has been found in bacterial infections, such as in gingival inflammation (20, 26) and osseous inflammation associated with lesions of endodontic origin (13). Despite the capacity of mononuclear phagocytes to serve a positive role in the host response to microbial invasion, their products can also have a destructive effect, as established in several chronic inflammatory diseases. In pulp and periodontal tissues infected with pathogenic bacteria, neutrophil infiltrates appear as the first line of de-

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fense against the bacterial insult. Because of their capacity to attract and activate neutrophils and monocytes, respectively, IL-8 and MCP-1 are thought to be important mediators in the inflammatory response to microbes. Peripheral blood mononuclear cells (PBMC) are a major source of chemokine production and are important components of the host defense. In addition, cytokines produced by mononuclear cells are important mediators for chemokine production by other cells, such as gingival fibroblasts, which respond to invading microorganisms and produce chemokines (5, 18).

To further understand the nature of acute and chronic inflammations caused by selected oral pathogens, we examined the differential expression of IL-8 and MCP-1 by human PBMC stimulated in vitro by the fungus *C. albicans*, the gram-positive bacterium *S. mutans*, and the gram-negative bacteria *P. gingivalis* and *A. actinomycetemcomitans*. The results indicate that these oral pathogens stimulate disparate concentration-specific patterns of IL-8 or MCP-1 gene expression and release.

MATERIALS AND METHODS

Microbes. A well-described type B isolate of *C. albicans* originally obtained from a patient with candidiasis (8) was grown in the yeast phase on Sabouraud dextrose agar at 25°C. Fungi were harvested after 4 days of growth, washed five times in phosphate-buffered saline (PBS), heat killed at 60°C for 30 min, and stored at 4°C. *S. mutans* was grown in Todd-Hewitt broth (Becton Dickinson, Cockeysville, Md.). Strains OMZ175 and LM7 were obtained from Frank Oppenheim (Boston University, School of Dental Medicine). *A. actinomycetemcomitans* was grown in enriched tryptic soy broth containing sodium carbonate, L-cysteine, hemin, and vitamin K1. Strains Y4 and ATCC29533 were obtained from Thomas Van Dyke (Boston University, School of Dental Medicine). *P. gingivalis* was cultured in enriched Todd-Hewitt broth containing the same supplements as described above. Strains A7A1-28, and 381 were obtained from Frank Oppenheim. Strain W50 was obtained from Thomas Van Dyke. Except where otherwise noted, strains OMZ175, Y4, and A7A1-28 were used for all studies. *A. actinomycetemcomitans* and *P. gingivalis* were cultured under strictly anaerobic conditions (85% N_2, 10% H_2, and 5% CO_2). The bacteria used in the study were at early to middle log phase. Overgrowth of microbes precluded using live organisms, so bacteria were heat killed by boiling for 10 min and then washed three times with PBS. The amount of bacteria was quantitated with a spectrophotometer according to a standard curve established by colony formation on bacterial plates. Lipopolysaccharide (LPS) was from *Escherichia coli* O111:B4.

Mononuclear cell stimulation. Blood was obtained from healthy volunteers by venipuncture and heparinized, and the PBMC were isolated from leukocyte-rich buffy coat following centrifugation over a gradient of Ficoll-Hypaque. Autologous serum was obtained by centrifugation of nonheparinized venous blood at the same time. PBMC (5×10^6 to 10×10^6) were incubated in polypropylene cell culture tubes containing the indicated stimulus in a final volume of 3 ml of RPMI supplemented with 2% autologous serum. All solutions and reagents had nondetectable endotoxin levels (<30 pg/ml) as determined by the *Limulus* amebocyte lysate assay (Sigma). After 1 to 21 h of stimulation, PBMC and supernatants were harvested for later Northern blot (RNA blot) analysis and chemokine enzyme-linked immunosorbent assay (ELISA), respectively.

Northern blots. Total RNA was isolated from PBMC by acid guanidinium thiocyanate-phenol-chloroform extraction, using a kit (TRI-Reagent; Leedo Medical Laboratory, Houston, Tex.). For each sample point, 2 to 5 μg of RNA was separated in a 1.2% agarose-formaldehyde gel and transferred to membranes (Zetabind). Blots were then hybridized with MCP-1 (E. Appella, Laboratory of Cell Biology, National Cancer Institute, Bethesda, Md.) or IL-8 (M. Fenton, Boston University Medical Center, Boston, Mass.) specific cDNA ^{32}P-labeled probes. Probes were labeled by random priming with the Klenow fragment and [^{32}P]dCTP. Hybridized probes were visualized by autoradiography.

Sandwich ELISAs for MCP-1 and IL-8. Monoclonal antibody hybridoma (E11) and polyclonal rabbit antibody for MCP-1 were donated by Edward Leonard (National Institutes of Health) and Anthony Valente (San Antonio, Tex.), respectively. Monoclonal antibody hybridoma (EL-NC-1S) and polyclonal rabbit antibody for IL-8 were purchased from the American Type Culture Collection and Endogen (Cambridge, Mass.), respectively. Horseradish peroxidase-labeled goat anti-rabbit antibody was purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, Md.). ELISAs were performed exactly as described previously (17, 25), using the horseradish peroxidase color development kit (Kirkegaard & Perry). The MCP-1 and IL-8 ELISAs were sensitive to ≤30 pg/ml and ≤300 pg/ml, respectively. Recombinant human MCP-1 and IL-8 were used as standards (Pepro Tech Inc, Rocky Hill, N.J., and R&D Systems, Minneapolis, Minn., respectively).

LDH assay. Lactate dehydrogenase (LDH) release as a measure of cell vitality was determined in supernatants of stimulated cells by using a commercial kit (TOX-7; Sigma).

RESULTS

Dose-dependent effects of microbes on MCP-1 and IL-8 release. In order to determine the effective doses of microbes needed to induce PBMC to produce MCP-1 or IL-8, dose-response experiments were carried out. PBMC were stimulated with the oral pathogens for 21 h, culture supernatants were collected, and IL-8 and MCP-1 release was determined by ELISA. In preliminary studies, it was determined that the number of microbes per PBMC needed for optimal stimulation varied widely between the tested microbes (data not shown). Based on these studies, three ratios of microbes to PBMC were chosen for subsequent experiments (Fig. 1). Release of MCP-1 followed a similar pattern to that seen with IL-8 release in PBMC stimulated with *C. albicans*, *P. gingivalis*, and *S. mutans*. Thus, release of both chemokines was greatest with the highest dose of *C. albicans* and *P. gingivalis* and the intermediate dose of *S. mutans*. In contrast, the amount of MCP-1 release was greatest with the lowest dose of *A. actinomycetemcomitans* while IL-8 production was maximally induced by the highest dose of *A. actinomycetemcomitans*. Compared with the other tested microbes, *S. mutans* induced the least amount of IL-8 protein release.

Parallel samples from the experiment depicted in Fig. 1 were collected at 4 h, and Northern blot analysis was used to determine the dose effect of microbes on MCP-1 and IL-8 mRNA expression (Fig. 2). In general, mRNA expression seen at 4 h directly correlated with protein release over 21 h. Relatively low concentrations of microbes induced high levels of MCP-1 mRNA, while higher concentrations of microbes were needed to maximally induce IL-8. At ratios of 0.05 and 0.02 microbe per PBMC, *C. albicans*, *S. mutans*, and *A. actinomycetemcomitans* stimulated peak expression of MCP-1, while 10 to 100 times more microbes were required for maximum IL-8 expression. Compared with the other tested microbes, a large number of *P. gingivalis* organisms, 25 and 250 microbes per PBMC, were required for maximal induction of MCP-1 and IL-8, respectively. The ratios of the microbes shown in Fig. 2 were chosen based on preliminary experiments, in which a 10-times-higher ratio of each microbe caused significant cytotoxicity to PBMC (see below) and a lower ratio did not stimulate a higher level of MCP-1 or IL-8 expression.

Cell viability. Prompted by our observation that the yield of total RNA isolated from PBMC was decreased after incubation with large numbers of microbes, cytotoxicity tests using LDH release as a marker of cell viability were carried out. Initial studies showed that at ratios of microbes to PBMC of 20:1 for *A. actinomycetemcomitans*, 5:1 for *C. albicans*, and 5:1 for *S. mutans*, considerable cell death occurred during the 21-h incubation period (data not shown). In general, in the range of microbe-to-PBMC ratios used in the chemokine experiments, LDH release was not significantly different from that seen in controls without microbes (Table 1). The exception was *S. mutans*, which at a ratio of 0.5:1 caused a significant amount of damage to PBMC. Although overall not statistically significant, at a ratio of 2 to 1 (microbe:PBMC), *A. actinomycetemcomitans* exhibited a highly variable degree of cytotoxicity between donors (range, 4 to 30%). Overall, dose- and microbe-dependent variations in MCP-1 and IL-8 production could not be attributed to changes in cell vitality.

Time-dependent induction of MCP-1 and IL-8 release and gene expression by oral microbes. Using the optimal microbe-to-PBMC ratios which stimulated MCP-1 and IL-8 release,

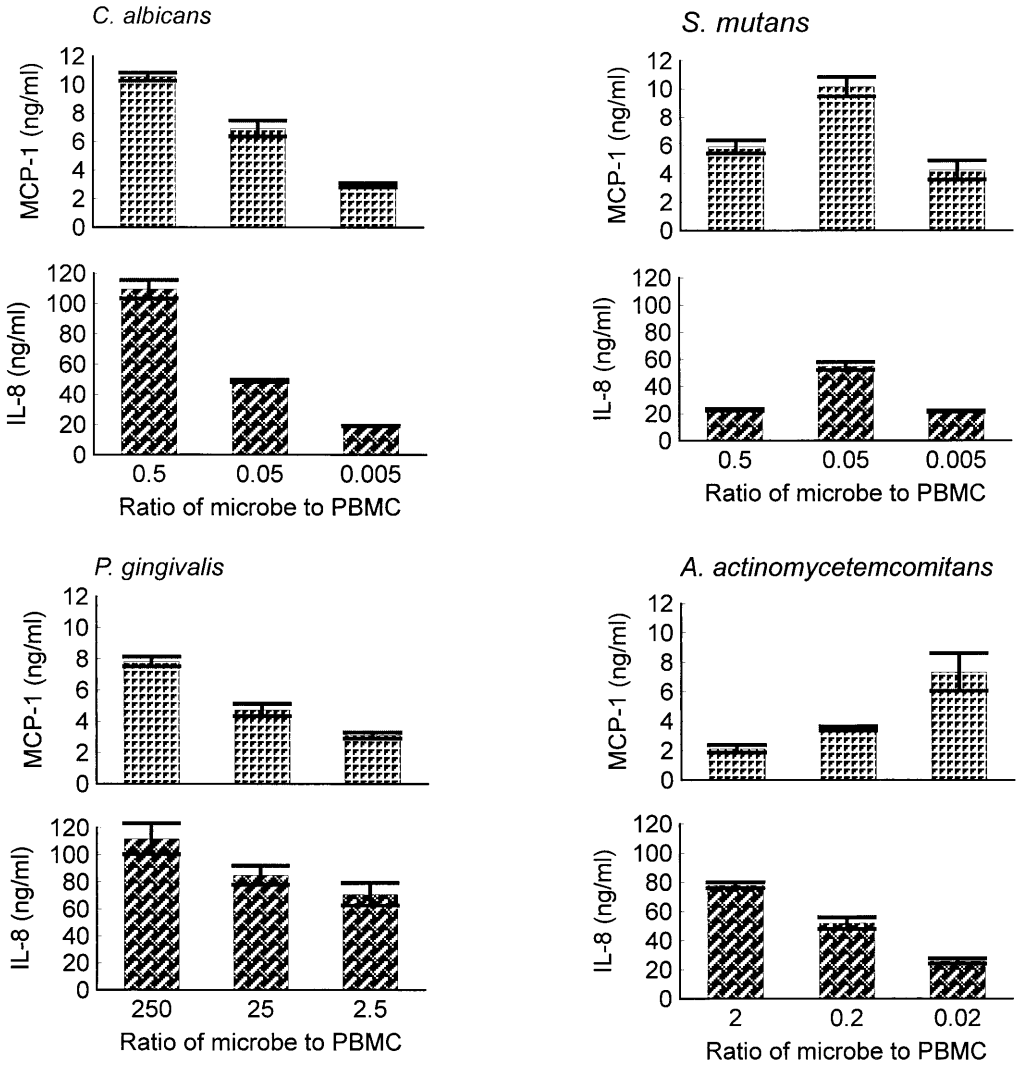

FIG. 1. Dose-dependent effects of microbes on MCP-1 and IL-8 protein release. PBMC were stimulated by the indicated ratios of microbes (*C. albicans*, *S. mutans*, *P. gingivalis*, and *A. actinomycetemcomitans*) to PBMC. After 21 h, the supernatants were collected and chemokine release was analyzed by ELISA. Spontaneous release from nonstimulated cells was 1 ng/ml for MCP-1 and 13 ng/ml for IL-8. Results shown are representative of three experiments.

MCP-1 protein release was low at 4 h and increased significantly after 21 h of stimulation compared with levels seen in unstimulated cells (Fig. 3a). In contrast, the amount of IL-8 protein release was significantly enhanced after 4 hours of stimulation with all tested microbes except for *S. mutans* and continued to increase over the 21-h stimulation (Fig. 3b). LPS (1 μg/ml) induced significant amounts of IL-8 but not MCP-1 protein release at 21 h of stimulation.

In order to determine the kinetics of MCP-1 and IL-8 mRNA induction, Northern blot analysis was performed on RNA extracted from PBMC that had been stimulated by the indicated microbes for 1, 4, 8, and 21 h (Fig. 4). MCP-1 mRNA levels were not detectable at 1 h and peaked at 4 to 8 h but were back at or near baseline levels at 21 h. In contrast, IL-8 mRNA levels were detectable within 1 h and reached peak levels at 4 h. Expression of IL-8, but not MCP-1, increased significantly over time even without microbial stimulation. This is likely due to higher levels of spontaneous production of IL-8 and is consistent with the observations of others (7).

Bacterial strain-related differences. In the final sets of experiments, we sought to determine whether data obtained using our bacterial isolates could be generalized to other strains within the same species (Fig. 5). Accordingly, MCP-1 protein release from PBMC stimulated by three *P. gingivalis* strains (A7A1-28, W50, and 381) and two strains each of *A. actinomycetemcomitans* (Y4 and ATCC29533) and *S. mutans* (OMZ175 and LM7) was determined. The pattern of dose-dependent effects on MCP-1 production was very similar between strains for the same bacteria. For all strains tested, higher ratios of *P. gingivalis* to PBMC were required to stimulate maximum production of MCP-1 than were needed for *A. actinomycetemcomitans* and *S. mutans.*

DISCUSSION

The presence of PMNs and monocytes at inflammatory sites reflects whether a lesion is acute or chronic and has a considerable impact on the continuing development of the host response. Chemokines are critical modulators of leukocyte recruitment. The data presented here relate to the induction and release of the chemokines MCP-1 and IL-8 from PBMC stimulated with clinically significant oral pathogens. Expression of

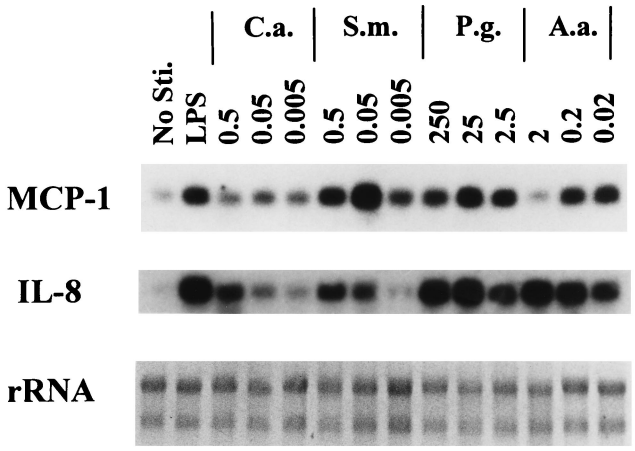

FIG. 2. Dose-dependent effects of microbes on MCP-1 and IL-8 mRNA induction. PBMC were stimulated under the same conditions as indicated for Fig. 1 for 4 h. Total RNA was isolated, and mRNA was analyzed by Northern blot. Nonstimulated cells (No sti.) served as a negative control. LPS was used at a concentration of 1 μg/ml. Results shown are from the same experiment as used for Fig. 1 and are representative of three experiments.

MCP-1 and IL-8 at both the mRNA and protein levels varied in a dose-dependent manner. The concentration of microbes required for maximum gene induction of MCP-1 was less than that for IL-8. In this regard, the limiting factor in microbial induction of IL-8 appears to be the cytotoxicity of the microbes tested. However, at the protein level, this difference in maximum induction concentrations for MCP-1 and IL-8 was not consistently observed, except in the case of *A. actinomycetemcomitans*, for which a 100-fold disparity was observed between the concentrations required for maximum MCP-1 and IL-8 gene induction. This disparity was also seen when chemokine protein release was measured. For other microbes tested, less prominent differences were seen in doses required for maximum induction of MCP-1 versus IL-8. This result suggests that the size of the microbial inoculation may affect the expression of a given chemokine. Thus, whether a lesion is acute or chronic may be influenced by the specific microbial infection and the dose of the inoculum.

Our studies also demonstrated considerable variation between microbes in their potency for stimulating chemokine production. *C. albicans* and *A. actinomycetemcomitans* stimulated MCP-1 and IL-8 production at an approximately 100- to 1,000-fold-lower microbe-to-PBMC ratio compared with *P. gingivalis*. However, the patterns of dose-dependent effects on MCP-1 production were very similar between strains for the same bacteria. For all strains tested, higher ratios of *P. gingivalis* to PBMC were required to stimulate maximum production of MCP-1 than with *A. actinomycetemcomitans* and *S. mutans.*

TABLE 1. Microbe-induced PBMC cytotoxicity[a]

Stimulus	Ratio of microbe to PBMC	% Damaged cells
None		6.2 ± 3.5
LPS, 1 μg/ml		13.9 ± 1.9
C. albicans	0.5	6.8 ± 1.5
	0.05	7.3 ± 3.7
	0.005	4.5 ± 1.2
S. mutans	0.5	22.2 ± 1.5*
	0.05	7.4 ± 1.8
	0.005	0.5 ± 0
P. gingivalis	250	9.3 ± 1.6
	25	7.2 ± 4.1
	2.5	4.3 ± 2.6
A. actinomycetemcomitans	2	16.7 ± 8.1
	0.2	12.6 ± 2.2
	0.02	3.3 ± 0.5

[a] PBMC were incubated with the indicated microbes for 21 h, following which the supernatants were collected and assayed for LDH. The results represent the mean ± standard error of three experiments. Statistical significance was determined by the Student *t* test (*, $P < 0.01$).

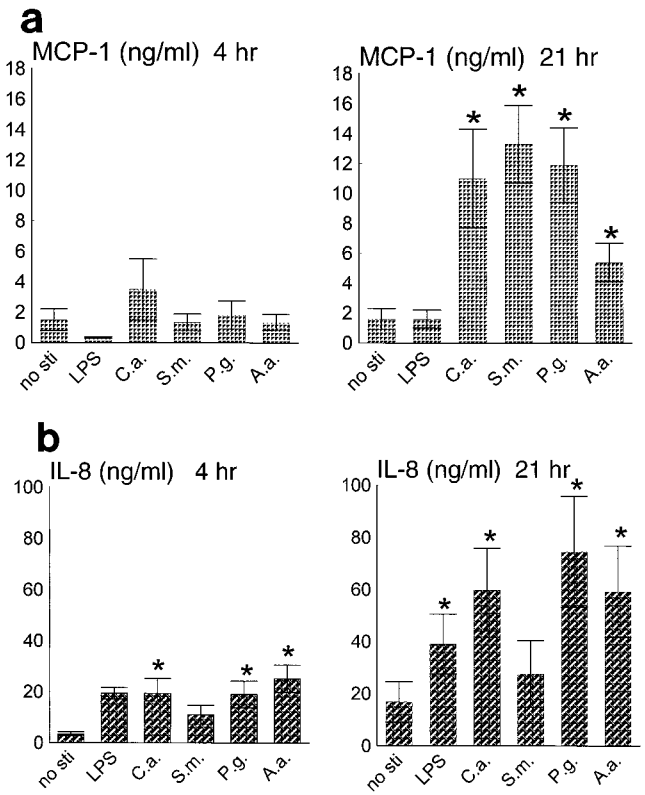

FIG. 3. Time-dependent induction of MCP-1 and IL-8 protein by different microbes. PBMC from seven donors were stimulated for 4 and 21 h at ratios of microbes to PBMC of 0.5:1 for *C. albicans*, 0.05:1 for *S. mutans*, 50:1 for *P. gingivalis*, and 0.2:1 (MCP-1) or 2:1 (IL-8) for *A. actinomycetemcomitans*. Nonstimulated cells (no sti) served as a negative control. LPS was used at a concentration of 1 μg/ml. The supernatants were collected, and MCP-1 (a) and IL-8 (b) release was analyzed by ELISA. *, increases significant compared with nonstimulated cells ($P < 0.02$).

The data from multiple donors (Fig. 3) indicate that *S. mutans* is as potent as *C. albicans* and *A. actinomycetemcomitans* in stimulation of MCP-1 but not IL-8 production. *S. mutans* failed to induce IL-8 protein release significantly above that seen in unstimulated cells. In contrast, other tested microbes were able to induce both MCP-1 and IL-8 mRNA and protein production. The transient expression of mRNA induced by *S. mutans* may explain the low levels of IL-8 released by PBMC. Our results demonstrate that relatively low numbers of *S. mutans* are capable of stimulating the production of a specific proinflammatory cytokine, MCP-1, by PBMC. It has been reported that exposure of the dental pulp to *S. mutans* causes experimental periapical inflammation (12, 15). Thus, even a small degree of contamination of the dental pulp by *S. mutans* could, by stimulating MCP-1 release, theoretically elicit an infiltration of monocytes, resulting in collateral tissue damage and necrosis. The different effects on MCP-1 and IL-8 induc-

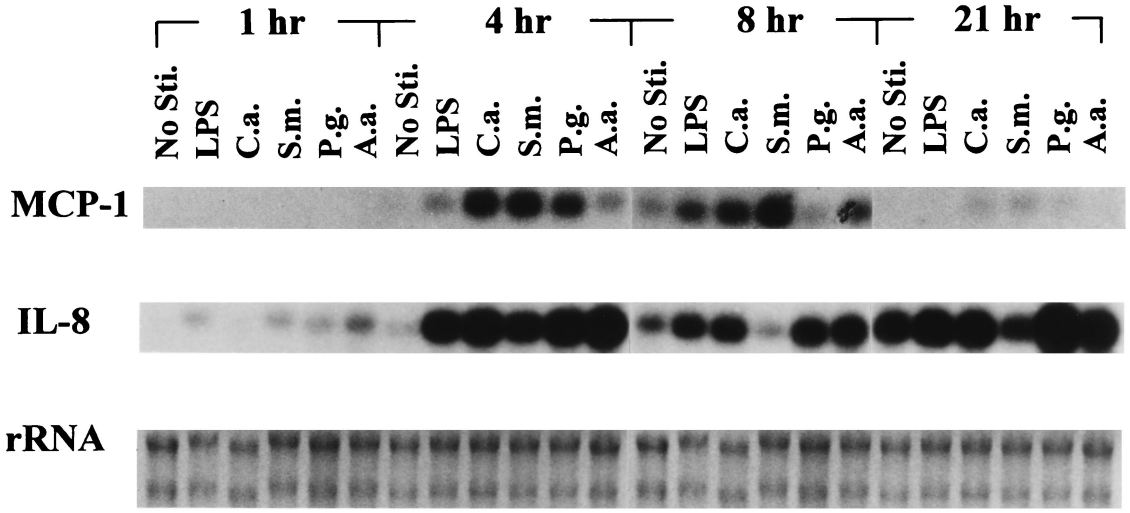

FIG. 4. Time-dependent induction of MCP-1 and IL-8 mRNA by different microbes. PBMC were stimulated for 1, 4, 8, and 21 h at ratios of microbes to PBMC of 0.5:1 for *C. albicans*, 0.05:1 for *S. mutans*, 50:1 for *P. gingivalis*, and 0.2:1 (MCP-1) or 2:1 (IL-8) for *A. actinomycetemcomitans*. Total RNA was isolated, and mRNA was examined by Northern blot analysis. Nonstimulated cells (No sti.) served as a negative control. LPS was used at a concentration of 1 μg/ml. Results shown are representative of two experiments.

tion by *S. mutans* demonstrate that different microbes may stimulate specific chemokine production and lead to recruitment of disparate leukocyte subpopulations.

P. gingivalis and *A. actinomycetemcomitans* are putative periodontal pathogens. Both *P. gingivalis* and *A. actinomycetemcomitans* stimulated MCP-1 and IL-8 production, although much higher concentrations of *P. gingivalis* were needed for maximum induction. As gram-negative organisms, *A. actinomycetemcomitans* and *P. gingivalis* contain endotoxin (14, 24). However, it is unlikely that endotoxin from these organisms was solely responsible for the chemokine induction seen in our experiments. First, a high concentration (1 μg/ml) of soluble LPS did not stimulate release of MCP-1 protein. Second, release of IL-8 from PBMC in response to a relatively high concentration of soluble LPS was less than that seen in response to *A. actinomycetemcomitans* and *P. gingivalis*. Future studies are planned to examine the microbial components responsible for inducing chemokine expression. Our data demonstrating lack of stimulation of MCP-1 by LPS is consistent with some, but not all, studies by other investigators (1, 9, 16).

Oral candidiasis is frequently associated with impaired cell-mediated immunity and neutropenia (11). In our studies, *C. albicans* was a potent inducer of relatively high levels of MCP-1 and IL-8. This observation may be significant since, in immunocompromised patients, *C. albicans* has been associated with inflammatory periodontal disease characterized pathologically by massive leukocyte infiltration (23). Thus, even low concentrations of *C. albicans* invading the gingival sulcus or other parts of the oral cavity may contribute to the inflammatory response by inducing chemokines which in turn recruit leukocytes. This leukocyte response likely accounts for the rarity of oral candidiasis in the immunocompetent host. However, in certain immunodeficient individuals prolonged inflammatory cell infiltration may contribute to tissue damage.

Thus, our findings indicate that specific oral microbial pathogens stimulate disparate patterns of MCP-1 and IL-8 gene expression and release in human PBMC. These findings may help explain the distinct immunopathology associated with different pathogens in the oral cavity. Moreover, these data support the concept that disease progression resulting from

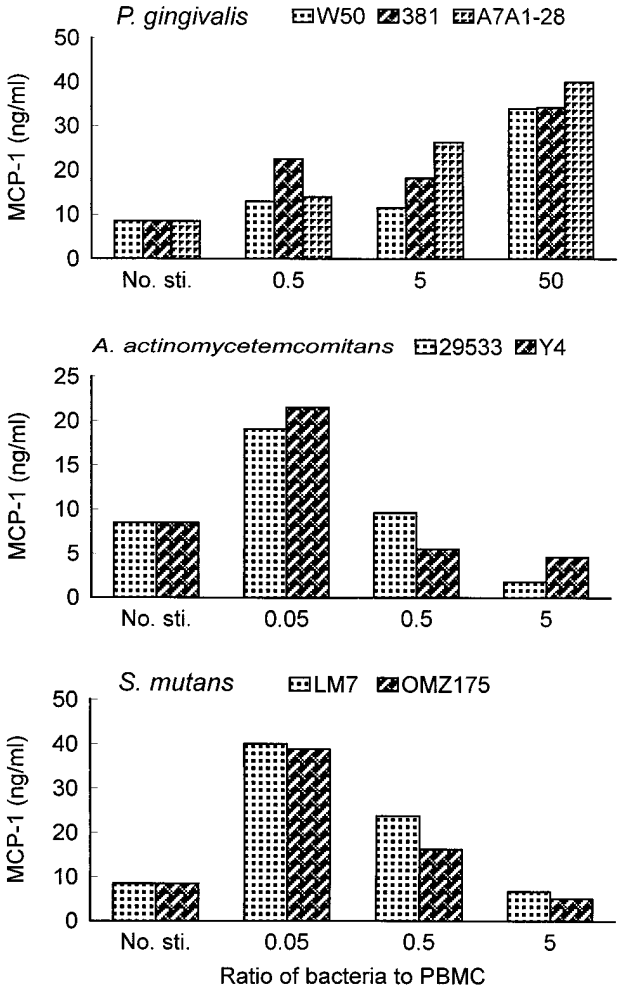

FIG. 5. Dose-dependent effects of different bacterial strains on MCP-1 protein release. PBMC were stimulated by the indicated ratios of bacteria (*P. gingivalis*, *A. actinomycetemcomitans*, and *S. mutans*) to PBMC. After 21 h, the supernatants were collected and MCP-1 release was analyzed by ELISA. Each datum point represents the mean of triplicate determinations. Results are representative of three donors.

microbial infection depends on the immunopathogenic properties of the pathogen, size of the inoculum, and host responses to the pathogen.

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