INFECTION AND IMMUNITY, Aug. 1993, p. 3533–3535
0019-9567/93/083533-03$02.00/0

Vol. 61, No. 8

Changes in Complement and Immunoglobulin G Receptor Expression on Neutrophils Associated with *Porphyromonas gingivalis*-Induced Inhibition of Phagocytosis

HIDEAKI TAI, TETSUO KOBAYASHI, AND KOHJI HARA*

Department of Periodontology, Niigata University School of Dentistry, 2-5274 Gakko-cho, Niigata, 951 Japan

Received 13 January 1993/Accepted 26 May 1993

We examined the phagocytic capacity and receptor expression on neutrophils stimulated with *Porphyromonas gingivalis* soluble products. Stimulated neutrophils had decreased phagocytic capacities and altered expression of CR1, CR3, FcγRII, and FcγRIII. For cases in which TLCK (*N*-α-*p*-tosyl-L-lysine chloromethyl ketone) neutralized the effects of the stimuli, the *P. gingivalis*-derived factors causing the phenomena seem to be trypsin-like proteases.

Periodontitis may present in several forms, but these forms display as a common trait a locally altered polymorphonuclear neutrophil (PMN) function (22–24). Adult periodontitis is the most frequent form, and its development is closely associated with the invasion of *Porphyromonas gingivalis*, which is known to inhibit phagocytic capacity (3, 26), upregulate complement receptor type 3 (CR3), and downregulate the *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) receptor on PMNs (20, 22, 23). Recent studies showed the importance of complement receptors and immunoglobulin G (IgG) receptors for PMN phagocytosis; the expressions of CR3 and both type II and type III IgG receptors (FcγRII and FcγRIII) on PMNs were reported as essential for both nonspecific and antibody-dependent cell cytotoxicity-mediated killing (5, 10, 13). Also, the relationship between PMN phagocytosis and dynamics of the expression of complement receptor type 1 and 4 (CR1 and CR4) has yet to be clarified. To further elucidate the mechanism by which *P. gingivalis* inhibits PMN phagocytosis, we stimulated PMNs with *P. gingivalis* culture supernatant dialysate (sup), *Streptococcus sanguis* sup, and fMLP, and then simultaneously assessed the PMN phagocytosis and expression of three complement receptors and two IgG Fc receptors by flow cytometry.

P. gingivalis 381 was used as a periodontopathic bacterial strain and *S. sanguis* ATCC 10556 was used as a control strain. Cells were grown in Trypticase soy broth (BBL, Cockeysville, Md.) supplemented with 0.5% yeast extract (Difco Laboratories, Detroit, Mich.) in 80% NO_2–10% CO_2–10% H_2 at 37°C up to the mid-logarithmic phase, collected by centrifugation (12,000 × *g*, 20 min, 4°C), and then dialyzed overnight with Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS). The sup from each culture was used as the bacterial stimulus for the experiments. The final protein content after addition to PMN suspension was 250 μg/ml, which was determined as the optimal subtoxic dose by a preliminary four-dose assay (15, 26).

PMNs were isolated from heparinized blood of healthy donors with dextran (Nakarai Chemical Co., Kyoto, Japan) sedimentation and centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden). Erythrocytes were eliminated by hypotonic lysis (1, 5). The PMN fraction was washed and resuspended in PBS containing 10 mM glucose (PBSg). The purity of the PMNs was routinely over 98%, and the viability was routinely over 97%. The PMN suspension was adjusted to 5×10^6 cells per ml and then was either preincubated with bacterial sups (1 h, 37°C, with shaking) or primed with 5 μg of cytochalasin B per ml (Sigma, St. Louis, Mo.) and stimulated with 10^{-6} M fMLP (Sigma) for 30 min at 37°C (12, 20). Negative controls were incubated with PBSg. After the washing, the cells were resuspended in either PBSg (phagocytic assay) or PBSg with 0.1% NaN_3 and 3 mM EDTA (receptor assay). Carboxylate monodisperse fluorescent beads (2-μm diameter) (Polyscience, Warrington, Pa.) were opsonized with normal human serum for 30 min at 37°C. The beads' suspension was adjusted to 5×10^7 beads per ml to reach a PMN/bead ratio of 1/10, and this was then added to the PMN suspensions for a 45-min incubation at 37°C. The samples were washed with PBSg prior to flow cytometric phagocytic assay (4, 19).

To assess possible parallelism between changes in phagocytic capacity and modulation of receptor expression, stimulated and unstimulated PMNs were reacted for 30 min at 4°C with fluorescein isothiocyanate-labeled anti-CR3 (Mo-1; Coulter Corp., Hialeah, Fla.), phycoerythrin-labeled anti-FcγRIII (Leu-11c; Becton Dickinson, San Jose, Calif.), anti-CR4 (FK24; Nichirei, Tokyo, Japan), and anti-CR1 and anti-FcγRII (IOT17 and IOT32; Immunotech, Marseilles, France). Samples reacted with unlabeled monoclonal antibodies were washed with PBSg containing 0.1% NaN_3–3 mM EDTA and were incubated for 30 min at 4°C with

TABLE 1. PMN phagocytic capacity stimulated by bacterial supernatants

Stimulus	% Phagocytes (mean ± SD)[a]	No. of phagocytosed beads (mean ± SD)[b]
PBS (control)	53.285 ± 14.34	1.345 ± 0.559
P. gingivalis sup	16.069 ± 3.266[c]	0.243 ± 0.07[c]
S. sanguis sup	56.131 ± 13.83	1.471 ± 0.588
fMLP	66.946 ± 15.171	1.842 ± 0.736

[a] Relative percentage of the phagocytosed cells to the total number of cells.
[b] Number of fluorescent beads phagocytosed by each PMN.
[c] Significantly different from PBS ($P < 0.001$).

* Corresponding author.

TABLE 2. Expression of PMN surface receptors stimulated by bacterial supernatants

Stimulus	Fluorescence intensity (mean ± SD) of:				
	CR1	CR3	CR4	FcγRII	FcγRIII
PBS (control)	56.228 ± 31.797	94.585 ± 22.663	244.899 ± 146.545	64.376 ± 6.303	86.681 ± 48.912
P. gingivalis sup	8.634 ± 8.457[a]	228.168 ± 28.397[a]	830.788 ± 234.052[a]	48.585 ± 7.369[a]	20.339 ± 16.74[a]
S. sanguis sup	186.984 ± 106.788[a]	262.147 ± 45.938[a]	1,060.682 ± 412.233[a]	66.531 ± 10.517	62.146 ± 39.911[b]
fMLP	126.976 ± 73.554[b]	343.273 ± 62.121[a]	1,363.721 ± 491.876[a]	62.851 ± 8.787	49.695 ± 28.282[b]

[a] Significantly different from PBS ($P < 0.001$).
[b] Significantly different from PBS ($P < 0.01$).

subclass-matched fluorescein isothiocyanate- or phycoerythrin-labeled goat anti-mouse Ig antibodies (American Qualex, La Miranda, Calif., and Tago, Burlingame, Calif., respectively). After reaction with antibodies, the samples were washed and analyzed with a FACScan flow cytometer (Becton Dickinson). Ten thousand cellular events were recorded. After gating of the PMN fraction, the phagocytic capacity was quantified as (i) the percentage of cells phagocytosing fluorescent beads to the total number of cells and (ii) the mean number of beads phagocytosed by a PMN. The upper limit of the assay was five phagocytosed beads per PMN (4, 19). For the receptor assay, the samples were analyzed for their green or red fluorescence intensity.

P. gingivalis-derived proteases are known to affect the local host immunity (11, 17, 25). We therefore verified whether protease inhibitors may neutralize the action of *P. gingivalis* sup. Before addition to PMN suspension, *P. gingivalis* sup was reacted with the three protease inhibitors leupeptin, antipain, and TLCK (Sigma) for 30 min at 37°C. The concentration of the protease inhibitors was 1 mM, which was determined to be optimal and subtoxic by a preliminary five-point assay. *P. gingivalis* sup thus reacted was used as a stimulus for flow cytometric assessment of CR1, CR3, FcγRII, and FcγRIII expression on PMNs. Inhibition of *P. gingivalis*-derived proteases by the inhibitors was expressed as a percentage relative to the results obtained with PBS alone: [(MFS − MFPBS)/MFPBS] × 100, where MFS and MFPBS are mean fluorescence intensity of receptor expression on PMNs stimulated with protease inhibitor-treated *P. gingivalis* sup and PBS alone, respectively. Results from a similar procedure, but with *S. sanguis* sup were used as a bacterial control. All data were analyzed by a paired *t* test. Differences were considered significant at $P < 0.01$.

P. gingivalis sup significantly inhibited PMN phagocytosis ($P < 0.001$), whereas *S. sanguis* sup and fMLP failed to cause significant decreases (Table 1). As shown in Table 2, CR3 and CR4 were upregulated by all stimuli. While *S. sanguis* sup and fMLP significantly enhanced CR1 expression, stimulation with *P. gingivalis* sup caused a decrease of CR1 expression ($P < 0.001$). *P. gingivalis* sup decreased the expression of both FcγRII and FcγRIII ($P < 0.001$), but *S. sanguis* sup and fMLP did not affect FcγRII expression. Also, the decrease in FcγRIII expression was greater with *P. gingivalis* sup than with *S. sanguis* sup and fMLP.

The effects of protease inhibitors on *P. gingivalis* sup are summarized in Table 3. Compared with untreated *P. gingivalis* sup, CR1 expression on PMN increased when stimulated with leupeptin- and antipain-treated *P. gingivalis* sup. TLCK also attenuated the *P. gingivalis* sup-induced CR1 downregulation, whereas the same protease inhibitor limited the extent of CR3 upregulation. Interestingly, both FcγRII and FcγRIII expression levels were increased by the addition of TLCK. FcγRIII expression was also increased by the addition of leupeptin.

The changes induced by *P. gingivalis* sup on PMN receptor expression differed from those of *S. sanguis* sup and fMLP. Although CR3 and CR4 were upregulated by all stimuli, *P. gingivalis* sup also decreased CR1 expression. While the upregulation of CR3 and CR4 can contribute to triggering of a rapid mobilization from the intracellular pool (16), decrease of CR1 by *P. gingivalis* sup not only suggests an impaired C3b and/or C4b clearance but also an impaired attachment of C3b-opsonized particles by *P. gingivalis*-exposed PMNs. Moreover, the weaker upregulation of CR3 by *P. gingivalis* sup than by the two other stimuli suggests a decreased PMN phagocytosis in the periodontitis lesion. *P. gingivalis* sup downregulated the expression of FcγRII, which is indispensable in IgG-mediated PMN killing and

TABLE 3. Changes in receptor expression of PMNs stimulated by bacterial sup and protease inhibitors

Bacterial stimulus + protease inhibitors	% Change in receptor expression of[a]:			
	CR1	CR3	FcγRII	FcγRIII
P. gingivalis sup	−14.26 (75.68 ± 21.92)	134.81 (236.11 ± 36.40)	−10.52 (62.81 ± 7.21)	−27.57 (69.30 ± 39.39)
+ Leupeptin (1 mM)	90.04 (166.83 ± 59.61)[b]	146.18 (247.61 ± 40.59)	9.06 (63.82 ± 8.25)	−19.59 (77.55 ± 43.22)[b]
+ Antipain (1 mM)	93.85 (173.43 ± 58.81)[b]	141.52 (242.86 ± 45.84)	6.42 (66.34 ± 8.23)	−21.08 (76.28 ± 43.12)
+ TLCK (1 mM)	8.02 (103.23 ± 31.82)	27.63 (129.17 ± 28.11)[c]	8.55 (76.39 ± 11.79)[c]	−12.00 (84.67 ± 46.12)[b]
S. sanguis sup	205.23 (283.16 ± 49.41)	155.30 (255.14 ± 33.70)	6.59 (75.66 ± 12.72)	17.83 (112.41 ± 59.25)
+ Leupeptin (1 mM)	201.34 (276.85 ± 56.78)	143.83 (244.15 ± 29.02)	3.98 (73.57 ± 9.59)	21.67 (114.85 ± 55.29)
+ Antipain (1 mM)	157.87 (237.33 ± 46.53)[c]	129.84 (230.03 ± 26.76)	6.75 (75.36 ± 11.47)	36.71 (126.16 ± 55.97)
+ TLCK (1 mM)	57.56 (150.56 ± 35.17)[c]	29.04 (128.70 ± 11.36)[c]	7.23 (75.41 ± 11.39)	1.34 (94.53 ± 40.82)

[a] The percentages are changes induced by the stimuli calculated with unstimulated (PBS) fluorescence as 0%. Values in parentheses are fluorescence intensities (means ± standard deviations). Positive percentages are increases from the reference value obtained with PMNs incubated with PBS alone. Negative values are decreases from the reference value.
[b] Significantly different from bacterial sup without protease inhibitor ($P < 0.01$).
[c] Significantly different from bacterial sup without protease inhibitor ($P < 0.001$).

antibody-dependent cell cytotoxicity (6, 10, 13). Because FcγRII is structurally stable, the decrease in such receptor may be attributed to two mechanisms: either *P. gingivalis* sup caused an internalization of FcγRII, or the stimulus blocked the intracellular mobilization of FcγRII (14).

FcγRIII is characterized by its binding to the cell surface by a phosphatidylinositol (PI) anchor (8, 9, 14, 18) and is suspected to work in concert with FcγRII in IgG-mediated killing by trapping the IgG complex, thus facilitating recognition by FcγRII (2, 13). *P. gingivalis* sup induced a decrease in FcγRII and FcγRIII expression levels while it inhibited PMN phagocytosis as well. Therefore, *P. gingivalis* may cause an impaired IgG-mediated PMN killing. FcγRIII is released from the PMN surface by a rupture of PI anchor by PI-specific phospholipase C produced either from PMNs or bacteria (9). Hence, the stimuli used in our study could have induced the synthesis of intracellular PI-specific phospholipase C by PMNs. Also, *P. gingivalis* sup induced the strongest FcγRIII downregulation; *P. gingivalis* may produce PI-specific phospholipase C.

Reports have shown that *P. gingivalis*-derived trypsin-like protease (7, 21, 27) can cleave C3, C5, and Igs (11, 17, 25). In our study, addition of TLCK attenuated the effect of *P. gingivalis* sup, which shows that *P. gingivalis*-derived protease would be capable of inducing changes in PMN surface receptor expression levels. Also, leupeptin and antipain tended to restore FcγRIII expression. These differential effects of protease inhibitors suggest that *P. gingivalis* secretes several different proteases, but the issue should be further assessed.

Thus, *P. gingivalis* not only inhibited PMN phagocytosis but also changed the expression of CR1, CR3, FcγRII, and FcγRIII, which appeared to be induced by proteases. The consequence would be that decreased CR1 expression on PMNs induced by *P. gingivalis* sup leads to an impaired attachment of C3b-opsonized particles and hence is relevant to the inhibited phagocytic capacity. Decrease in the two Fc receptors expressed on normal PMNs suggests reduced antibody-dependent cell cytotoxicity and phagocytic capacity of IgG-opsonized particles, which we are currently assessing with IgG-coated beads.

We are grateful to Tadatoshi Kuratsuji of the National Children's Medical Research Center, Tokyo, Japan, for his scientific guidance. Special thanks are also due to Takashi Suzuki of our Department for helpful discussions throughout the study.

REFERENCES

1. **Berger, M., J. O'Shea, A. S. Cross, T. M. Folks, T. M. Chused, E. J. Brown, and M. M. Frank.** 1984. Human neutrophils increase expression of C3bi as well as C3b receptors upon activation. J. Clin. Invest. **74**:1566–1571.
2. **Brunkhorst, B. A., G. Strohmeier, K. Lazzari, G. Weil, D. Melnick, H. B. Fleit, and E. R. Simons.** 1992. Differential roles of FcγRII and FcγRIII in immune complex stimulation of human neutrophils. J. Biol. Chem. **267**:20659–20666.
3. **Cutler, C. W., J. R. Kalmar, and R. R. Arnold.** 1991. Phagocytosis of virulent *Porphyromonas gingivalis* by human polymorphonuclear leukocytes requires specific immunoglobulin G. Infect. Immun. **59**:2097–2104.
4. **Dunn, P. A., and H. W. Tyrer.** 1981. Quantitation of neutrophil phagocytosis, using fluorescent latex beads. J. Lab. Clin. Med. **98**:374–381.
5. **Gordon, D. L., G. M. Johnson, and M. K. Hostetter.** 1986. Ligand-receptor interactions in the phagocytosis of virulent *Streptococcus pneumoniae* by polymorphonuclear leukocytes. J. Infect. Dis. **154**:619–626.
6. **Graziano, R. F., and M. W. Fanger.** 1987. FcγRI and FcγRII on monocytes and granulocytes are cytotoxic trigger molecules for tumor cells. J. Immunol. **139**:3536–3541.
7. **Grenier, D., G. Chao, and B. C. McBride.** 1989. Characterization of sodium dodecyl sulfate-stable *Bacteroides gingivalis* proteases by polyacrylamide gel electrophoresis. Infect. Immun. **57**:95–99.
8. **Huizinga, T. W. J., D. Roos, and A. E. G. K. von dem Borne.** 1990. Neutrophil Fc-γ receptors: a two-way bridge in the immune system. Blood **75**:1211–1214.
9. **Huizinga, T. W. J., C. E. van der Schoot, C. Jost, R. Klaassen, M. Kleijer, A. E. G. K. von dem Borne, D. Roos, and P. A. T. Tetteroo.** 1988. The PI-linked receptor FcRIII is released on stimulation of neutrophils. Nature (London) **333**:667–669.
10. **Indik, Z., C. Kelly, P. Chien, A. I. Levinson, and A. D. Schreiber.** 1991. Human FcγRII, in the absence of other Fcγ receptors, mediates a phagocytic signal. J. Clin. Invest. **88**:1766–1771.
11. **Kilian, M.** 1981. Degradation of immunoglobulins A1, A2, and G by suspected principal periodontal pathogens. Infect. Immun. **34**:757–765.
12. **Kuijpers, T. W., A. T. J. Tool, C. E. van der Schoot, L. A. Ginsel, J. J. M. Onderwater, D. Roos, and A. J. Verhoeven.** 1991. Membrane surface antigen expression on neutrophils: a reappraisal of the use of surface markers for neutrophil activation. Blood **78**:1105–1111.
13. **Kushner, B. H., and N-K. V. Cheung.** 1992. Absolute requirement of CD11/CD18 adhesion molecules, FcRII, and the phosphatidylinositol-linked FcRIII for monoclonal antibody-mediated neutrophil antihuman tumor cytotoxicity. Blood **79**:1484–1490.
14. **Leino, L., and E.-M. Lilius.** 1992. The up- and down-modulation of immunoglobulin G Fc receptors and complement receptors on activated human neutrophils depends on the nature of activator. J. Leukocyte Biol. **51**:157–163.
15. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
16. **O'Shea, J. J., E. J. Brown, B. E. Seligmann, J. A. Metcalf, M. M. Frank, and J. I. Gallin.** 1985. Evidence for distinct intracellular pools of receptors for C3b and C3bi in human neutrophils. J. Immunol. **134**:2580–2587.
17. **Schenkein, H. A.** 1988. Failure of *Bacteroides gingivalis* W83 to accumulate bound C3 following opsonization with serum. J. Periodontal Res. **24**:20–27.
18. **Selvaraj, P., W. F. Rosse, R. Silber, and T. A. Springer.** 1988. The major Fc receptor in blood has a phosphatidylinositol anchor and is deficient in paroxysmal nocturnal haemoglobinuria. Nature (London) **333**:565–567.
19. **Steinkamp, J. A., J. S. Wilson, G. C. Saunders, and C. C. Stewart.** 1982. Phagocytosis: flow cytometric quantitation with fluorescent microspheres. Science **215**:64–66.
20. **Sugita, N., T. Suzuki, H. Yoshie, N. Yoshida, M. Adachi, and K. Hara.** J. Periodontal. Res., in press.
21. **Tsutsui, H., T. Kinouchi, Y. Wakano, and Y. Ohnishi.** 1987. Purification and characterization of a protease from *Bacteroides gingivalis* 381. Infect. Immun. **55**:420–427.
22. **Van Dyke, T. E.** 1984. Neutrophil receptor modulation in the pathogenesis of periodontal diseases. J. Dent. Res. **63**:452–454.
23. **Van Dyke, T. E., E. Bartholomew, R. J. Genco, J. Slots, and M. J. Levine.** 1982. Inhibition of neutrophil chemotaxis by soluble bacterial products. J. Periodontol. **53**:502–508.
24. **Van Dyke, T. E., H. U. Horoszewicz, L. J. Cianciola, and R. J. Genco.** 1980. Neutrophil chemotaxis dysfunction in human periodontitis. Infect. Immun. **27**:124–132.
25. **Wingrove, J. A., R. G. DiScipio, Z. Chen, J. Potempa, J. Travis, and T. E. Hugli.** 1992. Activation of complement components C3 and C5 by a cysteine proteinase (gingipain-1) from *Porphyromonas (Bacteroides) gingivalis*. J. Biol. Chem. **267**:18902–18907.
26. **Yoneda, M., K. Maeda, and M. Aono.** 1990. Suppression of bactericidal activity of human polymorphonuclear leukocytes by *Bacteroides gingivalis*. Infect. Immun. **58**:406–411.
27. **Yoshimura, F., M. Nishikata, T. Suzuki, C. I. Hoover, and E. Newbrun.** 1984. Characterization of a trypsin-like protease from the bacterium *Bacteroides gingivalis* isolated from human dental plaque. Arch. Oral Biol. **29**:559–564.