

A Human Monoclonal Antibody Which Inhibits the Coaggregation Activity of *Porphyromonas gingivalis*

YOSHIMITSU ABIKO,^{1*} NAOMI OGURA,² UTAKO MATSUDA,² KAZUO YANAGI,³
AND HISASHI TAKIGUCHI¹

Departments of Biochemistry¹ and Oral Surgery,² Nihon University School of Dentistry at Matsudo,
Chiba 271, and National Institute of Health of Japan, Tokyo 164,³ Japan

Received 27 December 1996/Returned for modification 4 March 1997/Accepted 1 July 1997

A B-cell line producing a human monoclonal antibody (HuMAb) against a recombinant 40-kDa outer membrane protein (OMP) of *Porphyromonas gingivalis* was constructed by in vivo immunization of a severe combined immunodeficiency C.B.-17/Icr mouse, which had been injected with human peripheral blood lymphocytes, with recombinant 40-kDa OMP and subsequent Epstein-Barr virus immortalization of B cells isolated from the spleen of the mouse. This HuMAb inhibited coaggregation between *P. gingivalis* vesicles and *Actinomyces naeslundii* cells.

Porphyromonas gingivalis, which is considered an important pathogen in adult periodontitis (27), has several characteristics which make it a virulent pathogen, including production of proteolytic enzymes, adherence to buccal epithelial cells and saliva-coated hydroxyapatite, and coaggregation with a number of gram-positive bacteria (23). It has also been reported that the presence of dental plaque containing *Actinomyces* species and other gram-positive bacteria may be essential for the attachment and aggregation of *P. gingivalis* cells after introduction into the mouth (12, 23). The reports suggested that this attachment and colonization may contribute to various disease states, including periodontitis.

The bacterial surface, specifically the outer membrane, is important in attachment and aggregation. Some gram-negative oral bacteria can release microvesicles from the outer membrane which seem to consist of unmodified outer membrane protein (OMP) and to have various bacterial activities associated with them (10). *P. gingivalis* also releases extracellular vesicles, causing coaggregation with surrounding coinfecting microbial species (10), such as *Actinomyces naeslundii* (7) (strains isolated from humans and formerly named *Actinomyces viscosus* and *A. naeslundii* are now included in *A. naeslundii* [14]). We previously cloned an OMP gene from *P. gingivalis* 381 (2). This gene encodes a 40-kDa protein which is localized to vesicles (2) and is considered to be one of the *P. gingivalis* factors involved in coaggregation with *A. naeslundii*. Since the 40-kDa OMP has a molecular size similar to that of the fimbrial protein (28), hemagglutinins (24), and proteases (21) of *P. gingivalis*, we were interested in the relationship of our OMP to those known molecules. The amino acid composition of the OMP (2) and the nucleotide sequence (unpublished data) of the OMP gene were totally different from those of the fimbrial protein. Furthermore, the OMP did not express any protease activity against gelatin or albumin and casein used as protease substrates in zymography (data not shown), and a polyclonal antibody against the OMP failed to inhibit the hemagglutinin activity of vesicles (data not shown). These findings suggest that our OMP is a molecule distinct from the known proteins.

Recently, it has been reported that significantly less recolo-

nization by *P. gingivalis* of sites with severe periodontitis was found following topical application of a monoclonal antibody (MAb) against *P. gingivalis* (3). However, administration of MAb into the human body leads to the formation of anti-mouse antibodies which may diminish their effectiveness and produce adverse reactions because the MAb was mostly of murine origin (26). To develop a safe regimen for passive immunization therapy, several methods for the production of human MAbs (HuMAb) have been developed. A typical method for generation of HuMAb is immortalization of human B lymphocytes by hybridoma technology (13). However, only a few substances have been successful in establishing cell lines because peripheral blood lymphocytes (PBL) are in a resting state and their nuclei do not fuse with those of the myeloma partners (4). A novel technique has been reported whereby Epstein-Barr virus (EBV) can infect the human B lymphocytes through the membrane antigen CD21 (8), immortalizing these cells (16). However, B lymphocytes producing efficient HuMAb are difficult to obtain, since immunization of humans is not a practical proposition. In order to overcome this problem of low efficiency, we have adopted severe combined immunodeficiency C.B.-17/Icr (SCID) mice injected with human PBL. Because SCID mice are deficient in both T- and B-lymphocyte functions due to mutation of the *scid* gene associated with recombination (9), all of the major cell populations present in PBL are found in the lymphoid tissue and blood of SCID mice injected with PBL (PBL-SCID mice) (18, 20). Furthermore, PBL-SCID mice show spontaneous secretion of human immunoglobulin (Ig) whereby a specific human antibody response is inducible following immunization with a specific antigen (20).

We report here the establishment and characterization of a HuMAb against a 40-kDa OMP of *P. gingivalis* which inhibits coaggregation activity by in vivo immunization of PBL-SCID mice with recombinant 40-kDa OMP and EBV transformation of human B cells obtained from the spleens of these animals.

Establishment of an EBV-B-cell clone secreting Hab-omp1. Recombinant 40-kDa OMP was purified by the method of Kawamoto et al. (15). Peripheral blood was obtained with informed consent from a donor with a high level of serum antibody against 40-kDa OMP. On day 1, a SCID mouse (male, 7 weeks old) was given anti-asialo-GM₁ antibody (Wako Pure Chemical Industries Ltd., Osaka, Japan) intraperitoneally to reduce the NK cell activities. On day 4, PBL (10⁷ cells) were isolated with Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) and

* Corresponding author. Mailing address: Department of Biochemistry, Nihon University School of Dentistry at Matsudo, 2-870-1. Sakaecho-nishi, Matsudo, Chiba 271, Japan. Phone and fax: 0081 (47) 361-8880. E-mail: yabiko@mascat.nihon-u.ac.jp.

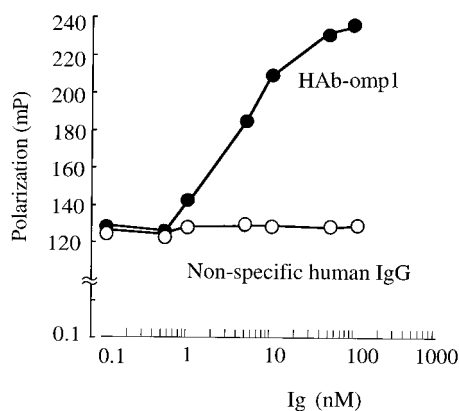


FIG. 1. Klotz plot of recombinant 40-kDa OMP and HAB-omp1 binding done with BEACON. Fluorescence polarization (P) is calculated as follows: $P = (\text{Int}_{\text{parallel}} - \text{Int}_{\text{perpendicular}}) / (\text{Int}_{\text{parallel}} + \text{Int}_{\text{perpendicular}})$, where Int is the intensity of the emission light parallel or perpendicular to the excitation light plane, as indicated (1 polarization unit = 1,000 mP units).

incubated with recombinant 40-kDa OMP (50 $\mu\text{g}/\text{ml}$) in 1 ml of RPMI medium (RPMI 1640 supplemented with 100 U of penicillin G/ml, 250 ng of amphotericin B (Fungizone)/ml, and 50 μg of gentamicin/ml) at 37°C for 30 min. This mixture was then injected into SCID mice intraperitoneally. The mice received booster injections of 50 μg of recombinant 40-kDa OMP intraperitoneally on days 12, 24, and 30. On day 38, spleen cells ($10^7/\text{ml}$) were isolated from PBL-SCID mice, mixed with EBV suspension prepared from the culture medium of the EBV-producing marmoset cell line B95-8, and cultured in RPMI medium containing 10% fetal calf serum at 37°C under 5% CO_2 in air. After 24 h, 100 ng of cyclosporine/ml was added to the cultures to suppress T cells. After a further 24 h, the culture medium was changed to fresh medium. The cells were cultured in a CO_2 incubator for 2 weeks, during which period the medium was changed twice a week. Foci of EBV-infected B lymphoblastoid cells (EBV-B cells) were observed within 2 weeks. After 4 weeks, the EBV-B cells which produced anti-recombinant 40-kDa OMP antibodies were collected by the panning method of Levitz and Dupont (17) using recombinant 40-kDa OMP. The cells selected by panning were cloned by the limited dilution method. Specific anti-recombinant 40-kDa OMP antibodies were detected by enzyme-linked immunosorbent assay with recombinant 40-kDa OMP. Twenty-five Ig isotype clones were examined, all of which produced human IgG isotype (23 clones belonged to the IgG1 subclass and 2 clones belonged to the IgG2 subclass) (data not shown). The human IgG that was reactive against recombinant 40-kDa OMP, as determined by demonstrating the highest titer with enzyme-linked immunosorbent assay and Western blot analysis and thus having the highest inhibitory activity against coaggregation, was IgG2 and was named HAB-omp1 (data not shown). This clone was used for further studies reported in this paper.

Recombinant 40-kDa OMP-HAB-omp1 equilibrium binding constant. We examined the equilibrium binding constant of the interaction between HAB-omp1 and recombinant 40-kDa OMP with a fluorescence polarization system (BEACON; Pan Vera Corp., Madison, Wis.) as described by Abbott and Ball (1). Recombinant 40-kDa OMP (3 mg) was labeled with fluorescein by using a fluorescein amino labeling kit (Pan Vera) and purified by gel filtration with a Sephadex G-25 column eluted with 10 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl. HAB-omp1 was purified by protein A affinity column

chromatography. An equilibrium binding isotherm was constructed by titrating a fixed concentration of the fluorescein-labeled recombinant 40-kDa OMP with increasing amounts of HAB-omp1. Figure 1 shows a Klotz plot of recombinant 40-kDa OMP-HAB-omp1. The equilibrium binding constant (K_d) was calculated from the fitted curve as 4.4 nM. In contrast, nonspecific human IgG did not bind recombinant 40-kDa OMP.

Inhibition of coaggregation activity by HAB-omp1. Coaggregation assay of *P. gingivalis* 381 vesicles and *A. naeslundii* (formerly *A. viscosus* ATCC 19246) cells by flocculation slide and radioactivity assays was performed by the method described previously (12). The flocculation slide assay showed that *A. naeslundii* cells did not aggregate by themselves (Fig. 2A). In contrast, coaggregation was significant when *P. gingivalis* vesicles were added to the cell suspensions (Fig. 2B). The coaggregation activity of the vesicles with *A. naeslundii* cells was significantly inhibited by HAB-omp1 (Fig. 2C). However, nonspecific human IgG did not inhibit coaggregation activity (Fig. 2D). Radioactivity assay demonstrated significant inhibition of coaggregation by HAB-omp1, as shown in Fig. 3. However, nonspecific human IgG had no effect on coaggregation.

It has been reported that direct transformation of PBL with EBV produced mainly IgM isotype antibodies (19). In this study, we obtained 25 clones which produced anti-recombinant 40-kDa OMP antibodies by using the SCID mouse immunization system. All of the HuMAbs produced by EBV transformation grafted into SCID mice were the IgG isotype. Satoh et al. reported that human EBV-B lymphoblastoid cell lines established from spleen and thymus cells of PBL-SCID mice were mostly of the IgG class (25). The possibility of generating a primary immune response in PBL-SCID mice is uncertain (22), but a secondary response or specific IgG secretion has

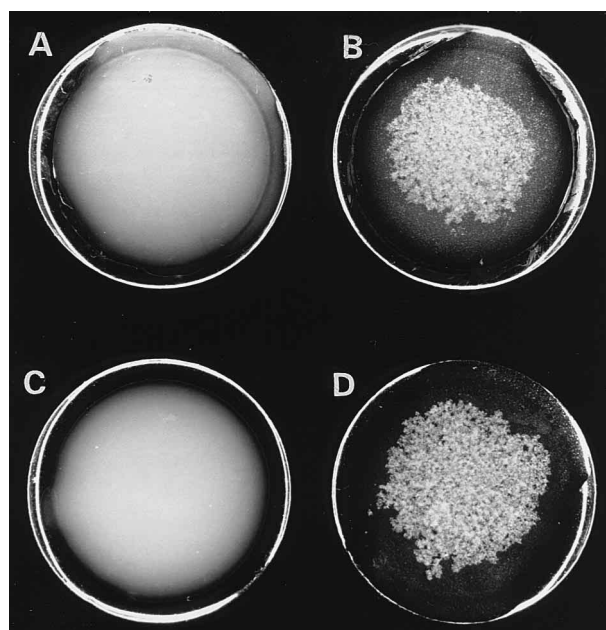


FIG. 2. Inhibition of coaggregation between *P. gingivalis* 381 vesicles and *A. naeslundii* cells by HAB-omp1 as determined by flocculation slide assay. (A) *A. naeslundii* cells (optical density at 550 nm [OD_{550}] = 1.0; 100 μl); (B) *P. gingivalis* vesicles (35 ng/500 μl) and *A. naeslundii* cells (OD_{550} = 1.0; 100 μl); (C) *P. gingivalis* vesicles (35 ng/500 μl) incubated with HAB-omp1 (100 μg) and *A. naeslundii* cells (OD_{550} = 1.0; 100 μl); (D) *P. gingivalis* vesicles (35 ng/500 μl) incubated with nonspecific human IgG (100 μg) and *A. naeslundii* cells (OD_{550} = 1.0; 100 μl).

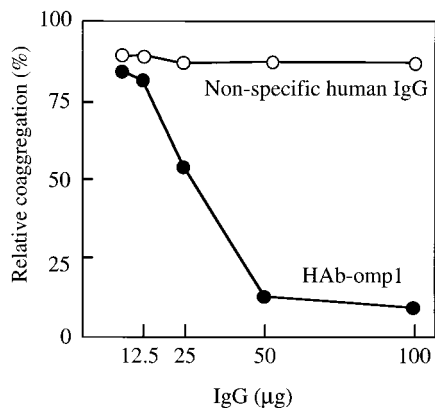


FIG. 3. Inhibition of coaggregation between *P. gingivalis* 381 vesicles (35 ng/500 µl) and *A. naeslundii* cells (optical density at 550 nm = 1.0; 100 µl) by HAB-omp1 as determined by radioactivity assay. Coaggregation was calculated relative to that in the absence of antibodies, which was taken as 100%.

been reported in several studies (5, 25). However, it was reported that most implanted human PBL were depleted by immune response with cytotoxic cells, mainly NK cells, in SCID mice (6). The surviving human PBL migrated to the tissues and then infiltrated into the tissues, proliferating in a suitable microenvironment of empty lymphoids (6). Therefore, administration of anti-asialo-GM₁ for reduction of NK cell activity would be necessary to improve the frequency of immortalization of human B cells (16).

On the other hand, although EBV transformation can be used for clonal expansion of B cells with concomitant production of antibodies, this technique has low immortalization efficiency. EBV-specific memory T cells may be responsible for controlling the level of EBV-positive B lymphocytes, which all healthy seropositive individuals carry for life after primary infection with the virus (11). Therefore, cyclosporine, which suppresses the immune activity of T cells, was added to the spleen cell cultures infected with EBV in this study.

It is well known that accumulation of bacteria on a tooth surface follows a sequence, beginning with gram-positive facultative species and shifting over time to gram-negative facultative and anaerobic species. *P. gingivalis* vesicles aggregated gram-positive bacteria, such as *A. naeslundii*, which are present in the earliest stages of dental plaque formation and are regarded as potential pathogens in periodontal diseases (7). In this study, we succeeded in obtaining HAB-omp1 capable of inhibiting the coaggregation between *P. gingivalis* vesicles and *A. naeslundii* cells by establishing a human B-cell line by PBL-SCID immunization and EBV transfection. This strategy is useful as a specific B-cell source and should make a major contribution to HuMAb technology. In further studies, we hope to characterize HAB-omp1 as being capable of inhibiting the *P. gingivalis* functions of fimbrially mediated adhesion, hemagglutination and protease activity, and coaggregation with many other partners among the oral flora.

This work was supported in part by Funds for Comprehensive Research on Aging and Health from the Ministry of Public Welfare of Japan (96A2303) and an Interdisciplinary General Joint Research Grant for Nihon University (DA96-013).

REFERENCES

- Abbott, A., and W. J. Ball, Jr. 1992. The inhibitory monoclonal antibody M7-PB-E9 stabilizes E2 conformational states of Na⁺, K⁺-ATPase. *Biochemistry* **31**:11236-11243.
- Abiko, Y., M. Hayakawa, H. Aoki, T. Kikuchi, H. Shimatake, and H. Takiguchi. 1990. Cloning of a *Bacteroides gingivalis* outer membrane protein gene in *Escherichia coli*. *Arch. Oral Biol.* **35**:689-695.
- Booth, V., F. P. Ashley, and T. Lehner. 1996. Passive immunization with monoclonal antibodies against *Porphyromonas gingivalis* in patients with periodontitis. *Infect. Immun.* **64**:422-427.
- Burnett, K. G., J. P. Leung, and J. Martinis. 1985. Human monoclonal antibodies to defined antigens, p. 113-133. In E. G. Engleman, S. K. H. Fong, J. Larrick, and A. Raubitschek (ed.), *Human hybridoma and monoclonal antibodies*. Plenum Press, New York, N.Y.
- Carlsson, R., C. Marthensson, S. Kalliomaki, M. Ohlin, and C. A. K. Borrebaeck. 1992. Human peripheral blood lymphocytes transplanted into SCID mice constitute an *in vivo* culture system exhibiting several parameters found in a normal humoral immune response and are a source of immunocytes for the production of human monoclonal antibodies. *J. Immunol.* **148**:1065-1071.
- Cesano, A., R. O'Connor, B. Lange, J. Finan, G. Rovera, and D. Santoli. 1991. Homing and progression patterns of childhood acute lymphoblastic leukemias in severe combined immunodeficiency mice. *Blood* **77**:2463-2474.
- Ellen, R. P., and D. A. Grove. 1989. *Bacteroides gingivalis* vesicles bind to and aggregate *Actinomyces viscosus*. *Infect. Immun.* **57**:1618-1620.
- Fingerroth, J. D., J. J. Weis, T. F. Tedder, J. L. Strominger, P. A. Biro, and D. T. Fearon. 1984. Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2. *Proc. Natl. Acad. Sci. USA* **81**:4510-4514.
- Fulop, G. M., and R. A. Phillips. 1990. The *scid* mutation in mice causes a general defect in DNA repair. *Nature* **347**:479-482.
- Grenier, D., and D. Mayrand. 1987. Functional characterization of extracellular vesicles produced by *Bacteroides gingivalis*. *Infect. Immun.* **55**:111-117.
- Henderson, E., G. Miller, J. Robinson, and L. Heston. 1977. Efficiency of transformation of lymphocytes by Epstein-Barr virus. *Virology* **76**:152-163.
- Hiratsuka, K., Y. Abiko, M. Hayakawa, T. Ito, H. Sasahara, and H. Takiguchi. 1992. Role of *Porphyromonas gingivalis* 40-kDa outer membrane protein in the aggregation of *P. gingivalis* vesicles and *Actinomyces viscosus*. *Arch. Oral Biol.* **37**:717-724.
- James, K., and G. T. Bell. 1987. Human monoclonal antibody production. *J. Immunol. Methods* **100**:5-40.
- Johnson, J. L., L. V. H. Moore, B. Kaneko, and W. E. C. Moore. 1990. *Actinomyces georgiae* sp. nov., *Actinomyces gerencseriae* sp. nov., designation of two genospecies of *Actinomyces naeslundii*, and inclusion of *A. naeslundii* serotypes II and III and *Actinomyces viscosus* serotype II in *A. naeslundii* genospecies 2. *Int. J. Syst. Bacteriol.* **40**:273-286.
- Kawamoto, Y., M. Hayakawa, and Y. Abiko. 1991. Purification and immunochemical characterization of a recombinant outer membrane protein from *Bacteroides gingivalis*. *Int. J. Biochem.* **23**:1053-1061.
- Kudo, T., S. Saijo, H. Saeki, N. Sato, T. Tachibana, and S. Habu. 1993. Production of a human monoclonal antibody to a synthetic peptide by active *in vivo* immunization using a SCID mouse grafted with human lymphocytes. *Tohoku J. Exp. Med.* **171**:327-338.
- Levitz, S. M., and M. P. Dupont. 1993. Phenotypic and functional characterization of human lymphocytes activated by interleukin-2 to directly inhibit growth of *Cryptococcus neoformans* *in vitro*. *J. Clin. Invest.* **91**:1490-1498.
- McCune, J. M., R. Namikawa, H. Kaneshima, L. D. Shultz, M. Lieberman, and I. L. Weissman. 1988. The SCID-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. *Science* **241**:1632-1639.
- Melamed, M. D., K. M. Thompson, T. Gibson, and N. C. Hughes-Jones. 1987. Requirements for the establishment of heterohybridomas secreting monoclonal human antibody to rhesus (D) blood group antigen. *J. Immunol. Methods* **104**:245-251.
- Mosier, D. E., R. J. Gulizia, S. M. Baird, and D. B. Wilson. 1988. Transfer of a functional human immune system to mice with severe combined immunodeficiency. *Nature* **335**:256-259.
- Nishikata, M., and F. Yoshimura. 1991. Characterization of *Porphyromonas (Bacteroides) gingivalis* hemagglutinin as a protease. *Biochem. Biophys. Res. Commun.* **178**:336-342.
- Nonoyama, S., F. O. Smith, and H. D. Ochs. 1993. Specific antibody production to a recall or a neoantigen by SCID mice reconstituted with human peripheral blood lymphocytes. *J. Immunol.* **151**:3894-3901.
- Okuda, K., J. Slots, and R. J. Genco. 1981. *Bacteroides gingivalis*, *Bacteroides asaccharolyticus* and *Bacteroides melaninogenicus* subspecies: cell surface morphology and adherence to erythrocytes and human buccal epithelial cells. *Curr. Microbiol.* **6**:7-12.
- Progulske-Fox, A., S. Tumwasorn, and S. C. Holt. 1989. The expression and function of a *Bacteroides gingivalis* hemagglutinin gene in *Escherichia coli*. *Oral Microbiol. Immunol.* **4**:121-131.
- Satoh, N., T. Kudo, H. Saeki, S. Saijo, T. Hasumi, H. Yoshida, S. Kobayashi, and S. Fujimura. 1995. A new and efficient method to generate human IgG

- monoclonal antibodies reactive to cancer cells using SCID-hu mice. *Immunol. Lett.* **47**:113-119.
26. **Schroff, R. W., K. A. Foon, S. M. Beatty, R. K. Oldham, and A. C. Morgan, Jr.** 1985. Human anti-murine immunoglobulin responses in patients receiving monoclonal antibody therapy. *Cancer Res.* **45**:879-885.
27. **Slots, J.** 1982. Importance of black-pigmented *Bacteroides* in human periodontal diseases, p. 27-45. *In* R. J. Genco and S. E. Mergenhagen (ed.), *Host-parasite interactions in periodontal disease*. American Society for Microbiology, Washington, D.C.
28. **Yoshimura, F., K. Takahashi, Y. Nodasaka, and T. Suzuki.** 1984. Purification and characterization of a novel type of fimbriae from the oral anaerobe *Bacteroides gingivalis*. *J. Bacteriol.* **160**:949-957.

Editor: J. R. McGhee