Application of Monoclonal Antibodies to the Detection of Black-Pigmented Bacteroides spp. in Subgingival Plaques by Immunoslot Blot Assay

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The aim of the present study was to assess the application of monoclonal antibodies to the detection of black-pigmented Bacteroides spp. in subgingival plaques by immunoslot blot assay. Subgingival plaque samples from adult periodontal patients were examined by immunoslot blot assay with monoclonal antibodies that specifically recognize Bacteroides gingivalis, Bacteroides intermedius serogroups I and II, and Bacteroides melaninogenicus. The assay can detect specifically these Bacteroides spp. in the subgingival plaques. Therefore, we investigated the distribution of these Bacteroides spp. in the subgingival plaques of patients classified by Russell's periodontal index. Reactivities of their plaques with monoclonal antibodies toward B. gingivalis and B. intermedius serogroup I were clearly related to the severity of the periodontal disease, but this was not the case with B. intermedius serogroup II and B. melaninogenicus. These results indicate that this immunoslot blot assay using monoclonal antibodies toward these Bacteroides spp. provides simple detection and monitoring of these organisms in periodontal patients.

Many recent studies (6, 10, 12, 17) have demonstrated that different forms of human periodontal disease are associated with specific microorganisms in subgingival plaque. For example, adult periodontal disease is associated with the presence of black-pigmented Bacteroides species, particularly B. gingivalis and B. intermedius (12). Localized juvenile periodontitis is also caused by Actinobacillus actinomycetemcomitans (2, 8, 19). Therefore, it is very important for diagnosis of periodontal disease to detect and monitor these periodontal pathogens in the plaque of patients. Presently, standard culture techniques and biochemical methods are widely used for the identification of periodontal pathogens. However, these methods are time consuming and have numerous technical difficulties owing to the fastidious nature of these organisms. Therefore, it is necessary to develop a specific and rapid method that detects and monitors these organisms in their plaques. For such an approach, several investigators (4, 5, 11, 20) have proposed the immunofluorescence assay using monoclonal and polyclonal antibodies having specificity against each organism, as well as the hybridization assay with DNA probes.

In the present study, we employed the immunoslot blot assay using monoclonal antibodies to detect or monitor rapidly and specifically black-pigmented Bacteroides spp. in subgingival plaques of adult periodontal patients.

MATERIALS AND METHODS

Subjects. Adult periodontal subjects (age range, 19 to 64 years) at Meikai University Hospital were classified into three groups by the periodontal index (PI) described by Russell (15): PI-1 (n = 59), PI = 1.0 to 3.0; PI-2 (n = 65), PI = 3.1 to 5.0; and PI-3 (n = 74), PI = 5.1 to 8.0. None of the subjects had received dental treatment or antibiotic therapy for at least 7 months before this study.

Sample collection. Subgingival plaques from each subject were obtained from the mesiobuccal surface of three selected teeth as described by Ramfjord (14). Clinical observations were recorded for each tooth as the plaque and gingival indices. First, supragingival plaques were removed with a sterile periodontal curette, and then subgingival plaques were collected with an excavator inserted to the base of the periodontal pocket. One loopful (catalog no. 254410; Nunc Co., Copenhagen, Denmark) of the collected plaques was transferred to microtubes containing 500 μl of sodium carbonate buffer (pH 9.6). The plaque samples (25 μl) were dispersed by pulsed sonication (Sonifier cell disrupter; Branson Sonic Power Co., Danbury, Conn.) for 10 s at 40 W. The dispersed samples were used for immunoslot blot assay as described below.

Bacterial strains. B. gingivalis 381, B. intermedius ATCC 25611 and ATCC 25261, Bacteroides melaninogenicus ATCC 25845, Bacteroides loescheii ATCC 15930, Bacterodes corporis ATCC 33547, Bacteroides denticola ATCC 33185, Bacteroides endodontalis ATCC 35406, Bacteroides asaccharolyticus ATCC 25260, A. actinomycetemcomitans Y4, Eikenella corrodens ATCC 23834, Capnocytophaga ochracea ATCC 27872, Fusobacterium nucleatum F7, and Treponema denticola ATCC 35405 were used in this study. EX-1 diffuse medium (9) was used for the cultivation of these bacterial cells except T. denticola. T. denticola was cultured in semisolid medium consisting of mycoplasma broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 10% rabbit serum and cocarboxylase. Sonicated extracts of bacteria were prepared as described previously (13). Protein contents of sonicated extracts were measured by the method described by Bradford (1).

Monoclonal antibodies. Monoclonal antibodies to B. gingivalis, B. intermedius serogroups I and II, and B. melaninogenicus were produced by techniques described earlier (7). B. gingivalis 381, B. intermedius ATCC 25611 and ATCC 25261, and B. melaninogenicus ATCC 25845 were used for...
immunization. Formalized cells of each species were injected into BALB/c mice, and the spleen cells of these immunized animals were collected and then fused with SP2/0-Ag14 myeloma cells by use of polyethylene glycol. Culture supernatants of hybridoma cells were tested against a sonicated extract of each bacterial species by enzyme-linked immunosorbent assay as described previously (3). The hybridoma cell lines were selected on the basis of species specificity and antibody activity and cloned by the limiting dilution procedure. Species specificity for each monoclonal antibody was evaluated from its immunoslot blot assay reactivity with the black-pigmented Bacteroides spp. The following monoclonal antibodies were selected for use in this study: monoclonal antibody BGF7, specific for B. gingivalis; BIF6 antibody, specific for B. intermedii serogroup I; BIF5 antibody, specific for B. intermedii serogroup II; and BMF4 antibody, specific for B. melaninogenicus. Culture supernatants of monoclonal antibody-producing cells were used for the immunoslot blot assay.

**Immunoslot blot assay.** Subgingival dental plaque samples (25 μl) suspended in 500 μl of sodium carbonate buffer or each sonicated bacterial extract (25 ng of protein) was blotted onto nitrocellulose paper (Bio-Rad; Bio-Rad Japan, Tokyo) apically to each well of an immunoslot blot apparatus (Hybri-slot manifold; Bethesda Research Laboratories, Gaithersburg, Md.). After blotting, the paper was treated for 30 min with 3% gelatin and washed with phosphate-buffered saline containing 0.02% (vol/vol) Tween 20 (PBS-Tween). After washing, the paper was treated for 60 min with culture supernatants of monoclonal antibody-producing cells or control SP2/0-Ag14 myeloma cells and then washed by shaking for 30 min with PBS-Tween. The treated paper was next incubated for 60 min with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Bio-Rad), washed, and then visualized by use of horseradish peroxidase color development reagent (Bio-Rad). Densitometric analysis of the reactivities was performed with a graphic analyzer (Shoni GA; Showa Denkoh, Tokyo, Japan). The reactivity was evaluated as positive when the intensity of a test sample was more than 0.5 with respect to an arbitrary value of 1 assigned to the intensity of the reactivity obtained for each positive control. The results were expressed as a percentage of the positive number to the total number of the subgingival dental plaques tested.

**RESULTS**

**Immunoslot blot assay using monoclonal antibodies against black-pigmented Bacteroides spp.** Figure 1 shows the results of immunoslot blot assay for reactivity of periodontal pathogens with four monoclonal antibodies (BGF7, BIF6, BIF5, and BMF4). Each sonicated bacterial extract was blotted onto nitrocellulose paper in wells of the immunoslot blot apparatus. The culture supernatant of SP2/0-Ag14 myeloma cells was used as a negative control throughout this study. The reactivities obtained by immunoslot blot assay showed strict specificity of these monoclonal antibodies toward the respective black-pigmented Bacteroides spp. used as immunogens (Fig. 1).

**Detection of black-pigmented Bacteroides spp. in subgingival plaques by immunoslot blot assay using monoclonal antibodies.** The dispersed subgingival plaques from periodontal subjects were blotted onto nitrocellulose paper in the wells of the immunoslot blot apparatus and then examined for their reactivities toward the four monoclonal antibodies. None of the samples were positive when tested with control supernatant and goat anti-mouse immunoglobulin G antibody conjugated with horseradish peroxidase, the secondary antibody (Fig. 2). Specific reactivities toward these monoclonal antibodies were observed in some samples of the subgingival plaques. These results show that immunoslot blot assay using monoclonal antibodies can detect specifically the black-pigmented Bacteroides spp., B. gingivalis, B. intermedii serogroups I and II, and B. melaninogenicus, in the subgingival plaques. Therefore, we next examined the distribution of these Bacteroides spp. in the subgingival plaques of adult periodontal subjects classified by Russell’s PI.

**Distribution of black-pigmented Bacteroides spp. in subgingival plaques of adult periodontal patients as detected by the immunoslot blot assay.** The subgingival plaques of adult periodontal patients were prepared, and to monitor and detect black-pigmented Bacteroides spp. in these plaques, the immunoslot blot assay was performed using the BGF7, BIF6, BIF5, and BMF4 monoclonal antibodies. BGF7 antibody reacted with 45 (25%) of the 177 subgingival plaques of PI-1 subjects tested (Fig. 3). And positive results obtained from those of PI-2 and PI-3 subjects were 55% (108 of 195) and 66% (147 of 222), respectively. In the immunoslot blot assay using BIF6 antibody, 66% (136 of 205) for B. melaninogenicus and 72% (173 of 222), respectively. However, in the case of BIF5 antibody, which identifies B. intermedii serogroup II, there was little difference in the percentage of positive results among PI-1, PI-2, and PI-3 subjects. Reactivities toward BMF4 antibody indicated that very few plaques contained B. melaninogenicus.

Next, we analyzed the relation between the reactivity of the plaques toward each monoclonal antibody and the depth of the subgingival pockets. Reactivities of plaques toward BGF7 and BIF6 antibodies were clearly related to the depth of the subgingival pocket (Fig. 4a and b). However, no such relation was observed for BIF5 and BMF4 monoclonal antibodies (Fig. 4c and d).

These results indicate that both B. gingivalis and B. intermedii serogroup I, but not B. intermedii serogroup II and B. melaninogenicus, in subgingival plaques of adult periodontal patients may increase in frequency with the severity of the disease.

**DISCUSSION**

We have shown herein that the immunoslot blot assay using monoclonal antibody is a useful tool for monitoring or detecting black-pigmented Bacteroides spp. in the subgingival plaques of adult periodontal patients. Also, since this assay recognizes these bacteria or their cell-specific antigens present in the plaques with high specificity, it may be possible to apply this assay to diagnosis of periodontal diseases associated with specific microorganisms.

Many workers have used standard cell culture and biochemical methods for the monitoring of periodontopathic bacteria in subgingival plaques. Since these methods have several technical difficulties, it is very important to develop rapid and simple assays for monitoring the organisms in the plaques. Some investigators (5, 18) have demonstrated by immunofluorescence with specific monoclonal and polyclonal antibodies the presence of periodontopathic bacteria, such as B. gingivalis, B. intermedii, Bacteroides forsythus, and A. actinomycetemcomitans. This method has several advantages for detecting and monitoring the bacteria, includ-
fundamental weakness of Reactivity against the culture necessity the laborious. Consequently, rapidity, quantification, and sensitivity. However, the fundamental weakness of the immunofluorescence assay is the necessity of microscopic examination. Therefore, when many samples must be examined, this assay becomes very laborious. Consequently, we decided to develop the immunoslot blot assay using species-specific monoclonal antibody as described herein.

We showed that our monoclonal antibodies against selected black-pigmented Bacteroides spp. react specifically with the respective cell extracts (Fig. 1). The monoclonal antibodies can directly detect black-pigmented Bacteroides spp. in subgingival plaques of adult periodontal patients without false-positive reactions (Fig. 2). These findings indicate that the immunoslot blot assay using species-specific monoclonal antibody can be applied as a useful tool for direct detection or monitoring of periodontopathic bacteria in the plaques. As yet, we do not know whether our monoclonal antibodies react with the intact cells in the plaques or with specific antigen materials from cells released into the plaques or with both.

It has been shown by standard cell culture and biochemical methods that B. gingivalis and B. intermedius are the predominant species isolated from subgingival plaques of adult periodontal patients (17). The results of the immunoslot blot assay for plaque analysis showed that the reac-

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**FIG. 1.** Evaluation of specificity of each monoclonal antibody against black-pigmented Bacteroides spp. by immunoslot blot assay. Sonicated bacterial extracts (25 ng of protein per well) were blotted onto nitrocellulose paper in wells of the immunoslot blot apparatus. Reactivity against the monoclonal antibody was measured by immunoslot blot assay as described in Materials and Methods. The control was the culture supernatant of SP2/0-Ag14 myeloma cells.

**FIG. 2.** Detection of black-pigmented Bacteroides spp. in subgingival plaques by immunoslot blot assay. Test samples (lanes 1 to 11) of subgingival plaques of adult periodontal patients and each sonicated bacterial extract as positive controls (lane 12) were blotted onto nitrocellulose paper in each well of the immunoslot blot apparatus. Sample and control reactivities toward the four monoclonal antibodies were assessed by the immunoslot blot assay as described in Materials and Methods. Cont., Control.
activity of the plaques toward BGF7 and BIF6 antibodies increased with severity of this disease but that reactivity toward BIF5 and BMF4 antibodies did not (Fig. 3). These results thus confirm those obtained by the above-mentioned methods, that B. gingivalis and B. intermedius serogroup I predominate in subgingival plaques of this disease. Therefore, the immunoslot blot assay with monoclonal antibody appears to be a promising procedure for detection and monitoring of black-pigmented Bacteroides spp. in subgingival plaques. Also, a high correlation between reactivities of the plaques toward BGF7 and BIF6 antibodies and the depth of subgingival pocket was demonstrated (Fig. 4). These observations may support the immunoslot blot assay as a useful tool for diagnosis and monitoring of adult periodontal disease.

In conclusion, this immunoslot blot assay using monoclonal antibodies against black-pigmented Bacteroides spp. provides simple and rapid detection and monitoring of these microorganisms in adult periodontal patients. Therefore, this assay is extremely valuable for epidemiological studies of periodontal disease.

LITERATURE CITED
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