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Production of Monoclonal Antibodies That Recognize Specific and Cross-Reactive Antigens of *Fusobacterium nucleatum*

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Monoclonal antibodies (MAbs) against the cell surface antigens of *Fusobacterium nucleatum* 263 were obtained by fusion of murine myeloma cells (P3-NSI/1-Ag4-1) with the splenocytes of BALB/c mice immunized with whole cells of *F. nucleatum* 263. Screening was performed using an enzyme-linked immunosorbent assay (ELISA) against the immunizing strain, *F. nucleatum* 263. Further selection was done using a bacterial panel consisting of *Bacteroides*, *Actinomyces*, *Streptococcus*, *Fusobacterium*, and *Escherichia* species. Twelve MAbs were selected on the basis of this screening procedure, seven of which reacted specifically with *F. nucleatum* 263. Two reacted with *F. nucleatum* 263 and ATCC 25586, and three reacted with *F. nucleatum* 263, ATCC 25586, and UQD-003 (a clinical isolate) and also cross-reacted with *Fusobacterium russii* ATCC 25533. The selected MAbs were then further characterized by absorption experiments with suspensions of intact whole bacterial cells, and the residual binding activity of the supernatants was determined in an ELISA. To determine whether the MAbs reacted with the same or different epitopes, pairs of MAbs were reacted together and independently in a checkerboard manner in an ELISA. The additive or nonadditive nature of the reactivity was determined. A competitive inhibition assay was performed using one labeled and selected unlabeled MAbs. The results of these experiments suggested some epitope sharing among the selected MAbs that reacted with a specific antigen on *F. nucleatum* and also shared cross-reactive antigens with the three strains of *F. nucleatum* and *F. russii*.

Specific bacterial species play a major role in the etiology of periodontal disease (10). *Fusobacterium nucleatum*, a gram-negative anaerobic bacterium, has frequently been isolated from the subgingival sites of advancing lesions in patients with chronic inflammatory periodontal disease (25, 28, 30). Furthermore, studies have shown the presence of serum immunoglobulin G (IgG) antibodies specific for *F. nucleatum* which were significantly raised in periodontally diseased patients when compared with healthy individuals (4, 22, 23). These findings indicate a close association of *F. nucleatum* with chronic inflammatory periodontal disease.

Periodontal disease has been shown to have an immunological basis (16, 26), and therefore the mechanisms which control the immune response need to be studied, in particular the response to the bacterial cellular antigens and their products. Recently, studies have focused on the immune responsiveness to *F. nucleatum* in human and animal models. Workers have shown that extracts from *F. nucleatum* have a suppressive effect on human peripheral blood lymphocytes in vitro (27) and are able to induce polyclonal B-lymphocyte activation in vitro (19). Yoshie et al. (34) demonstrated that in mice an extract of *F. nucleatum* had an adjuvant effect on humoral immunity, as indicated by an increase in plaque-forming cells, a suppressive effect on cell-mediated immunity, a reduction in cytotoxic T cells to an allograft transplant, and an enhancing effect on phagocytic functions, with increased carbon clearance rates and bacteriocidal and acid phosphatase activities. Enhancement of B-cell function and suppression of T cells were further demonstrated with mucosal presentation of intact *F. nucleatum* cells (13).

Studies of these immune responses may be made easier by using monoclonal antibodies (MAbs), which can be used to purify the target antigen(s) responsible for eliciting the response described above. In addition, it may be possible to study the control of B-cell responses to bacterial antigens via idiotypic networks (12). The purpose of this paper is to describe the production and characterization of MAbs that react with *F. nucleatum*. Twelve MAbs which reacted with specific and cross-reactive antigens of *F. nucleatum* are described.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used were as follows: *F. nucleatum* 263, *Actinomyces viscosus* T-14, and *Streptococcus sanguis* 36A (kindly donated by A. C. R. Tanner, Forsyth Dental School, Boston, Mass.); *F. nucleatum* ATCC 25586, *Fusobacterium necrophorum* ATCC 25286, *Bacteroides gingivalis* ATCC 33277, *Bacteroides intermedius* ATCC 25611, and *Fusobacterium russii* ATCC 25533 (kindly donated by D. Love, Department of Veterinary Pathology, University of Sydney, N.S.W., Australia); *Fusobacterium mortiferum* ATCC 25551 and *Escherichia coli* ATCC 25922 (obtained from BactiDisks, Medical Diagnostics, Springwood, Queensland, Australia); *Fusobacterium varium* NCTC 10560 (kindly donated by the State Health Department of Western Australia, Perth); and *F. nucleatum* UQD-003, which was isolated from a clinical source (Department of Social and Preventive Dentistry, University of Queensland Dental School, Brisbane, Queensland, Australia).

Media and cultivation. Stock cultures were maintained in liquid nitrogen in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) containing 10% glycerol. Bacteria were checked for purity by growth on CDC blood agar prepared from a Trypticase soy agar base (40 g/liter; BBL) supplemented with agar (5 g/liter), yeast extract (5 g/liter; GIBCO Diagnostics, Madison, Wis.), L-cysteine hydrochlo-

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ride (0.5 g/liter; BDH, Poole, England), menadione (1 μg/ml; Sigma Chemical Co., St. Louis, Mo.), hemin, bovine type (5 μg/ml; GIBCO), and 5% defibrinated laked horse blood (31). Cultures were incubated at 37°C in an atmosphere of 80% N_2, 10% CO_2, and 10% H_2 in anaerobic jars (BBL) for 3 to 5 days.

Bacteria were grown in brain heart infusion broth (37.0 g/liter; Difco Laboratories, Detroit, Mich.) supplemented with yeast extract (5 g/liter; GIBCO), hemin (5 μg/liter; Sigma), menadione (1 μg/ml; Sigma), L-cysteine hydrochloride (0.5 g/liter; BDH), sodium formate (2 g/liter), sodium fumerate (3 g/liter), resazurin (1 g/liter; Sigma), and DL-dithiothreitol (0.1 g/liter; Sigma). The broth was prepared, prereduced, and anaerobically sterilized as described by Holdeman et al. (11) by using the VPI Anaerobic Culture System (Bellco Glass, Inc., Vineland, N.J.). The bacteria were harvested by centrifugation (model TJ-6; Beckman Instruments, Inc., Fullerton, Calif.) at 1,730 × *g* for 30 min and washed twice in sterile Dulbecco phosphate-buffered saline (PBS) containing 10 mM EDTA, and the suspensions were stored at −20°C as 1-ml aliquots.

Immunization of mice. BALB/c mice (6- to 8-week-old females) were obtained from the University of Queensland Central Animal Breeding House. The mice were immunized intraperitoneally with 0.1 ml (10^9 CFU/ml) of a bacterial suspension of *F. nucleatum* 263 on days 0 and 14. The mice were tail bled on day 21, and antibody levels to *F. nucleatum* 263 were determined by an enzyme-linked immunosorbent assay (ELISA) (see below). Mice with the highest titer were selected and boosted on day 28 with 0.2 ml (10^9 CFU/ml) of a bacterial suspension split into two equal doses, one given intraperitoneally and the other given as an intracardial injection 4 to 5 h later.

Production of hybridomas. The technique used for the production of hybridomas was essentially that of Kohler and Milstein (15) with the modifications of Galfre et al. (9). The normal culture medium consisted of RPMI 1640 (Flow Laboratories, Sydney, Australia) supplemented with 10% heat-inactivated fetal bovine serum (Symbio Products, Brisbane, Australia), 2 mM L-glutamine (Flow), penicillin (50 IU/ml; Flow), and streptomycin (50 μg/ml; Flow). The nonsecreting mouse myeloma line P3-NSI/1-Ag4-1 (NSI) (17) was grown initially in RPMI 1640 containing 6-thioguanine (40 μg/ml; Sigma) to prevent revertants. NSI cells were then fused with immune mouse spleen cells at an NSI/spleen cell ratio of 1:5 in the presence of 50% (wt/vol) polyethylene glycol (1450D; Sigma). This was then diluted with serum-free RPMI 1640 at 37°C and centrifuged at 150 × *g* for 7 min. The cells were suspended (2×10^5 cells per ml) in RPMI 1640 containing hypoxanthine (13.6 μg/ml), aminopterin (0.176 μg/ml), thymidine (3.88 μg/ml; Sigma), 20% fetal bovine serum, and β-mercaptoethanol (5×10^{-5} mM) (HAT medium) (14). After fusion, the cells were plated out into 24-well cluster plates (flat bottom; Linbro; Flow) by adding 1 ml of cell suspension per well and were placed in a humidified incubator (5% CO_2 in air) at 37°C. After 7 days, 1 ml of HAT medium was added. Culture supernatants were monitored, and when a change to acid conditions was observed, 1 ml of medium was removed and replaced by 1 ml of fresh HAT medium. Screening procedures were performed after 10 days by an ELISA (see below). Hybridomas secreting antibodies with a specific reactivity to *F. nucleatum* were cloned at least twice by the limiting dilution method (35). The cloned cells were expanded and injected into pristane-primed mice (0.5 ml of 2,6,10,14-tetramethylpentadecane [Aldrich Chemical Co., Inc., Milwaukee, Wis.]). Culture supernatants and ascites were stored at −20°C, and cells were frozen in the presence of 4% dimethyl sulfoxide in fetal bovine serum and stored in liquid nitrogen.

Detection of MAbs directed against *F. nucleatum* 263. (i) Preparation of bacterium-coated microtrays. Bacterium-coated 96-well plates were prepared by using the method of Nachamkin et al. (21). Briefly, a suspension of washed bacteria in PBS at an optical density of 0.50 (model 100-60 spectrophotometer; Hitachi, Tokyo, Japan) at 750 nm was added to the wells of a 96-well plate (50 μl per well; flat bottom DP2; Disposable Products, Adelaide, South Australia, Australia). The plates were centrifuged at 1,500 × *g* for 5 min at 4°C; 100 μl of cold 0.25% glutaraldehyde in PBS was then carefully added to each well, and the plates were centrifuged for an additional 5 min. After 15 min at room temperature (RT) the PBS-glutaraldehyde was flicked out, and the wells were washed three times with PBS–0.5% (vol/vol) Tween 20 (PBS-T). Finally, the wells were filled with PBS-T containing 1.0% bovine serum albumin (CSL, Melbourne, Australia) and incubated for 2 h at RT to block nonspecific binding sites. The plates were then stored for up to 1 month at 4°C without loss of activity.

(ii) ELISA. The ELISA was performed by the method of Engvall and Perlmann (5) with the plates precoated with bacteria or antiserum at the appropriate dilution. The primary antibody (100 μl) was added to the wells, incubated for 60 min at RT or overnight at 4°C, and washed three times with PBS-T, and the excess moisture was tapped out; 100 μl of rabbit anti-mouse immunoglobulin conjugated to horseradish peroxidase (1:1,000) (RAM-HRPO; Dakoplasts) in PBS-T containing 0.1% bovine serum albumin was then added to all wells and incubated for 60 min at RT. The plates were washed four times with PBS-T and developed by the addition of 150 μl of activated substrate to each well. The substrate was activated immediately before use and was prepared by adding 10 μl of a 3% H_2O_2 solution to 10 ml of solution containing 1 ml of 2.5 mM *o*-toluidine (Eastman Kodak Co., Rochester, N.Y.) diluted in 9 ml of 100 mM citrate buffer (pH 4.5) with 0.025 mM EDTA. The reaction was stopped after 10 min by the addition of 3 M hydrochloric acid (50 μl per well). The A_{450} was recorded with a Titertek Uniscan (Flow).

(iii) Isotype and subclass determination. Rabbit anti-mouse immunoglobulin subclass-specific antisera (Miles Scientific, Div. Miles Laboratories, Inc., Melbourne, Australia) at a 1:2,000 dilution in 0.05 M carbonate buffer (pH 9.6) were used to coat 96-well plates (Immunoplate IIF 442404; Nunc, Roskilde, Denmark). This coating dilution of antiserum was determined by block titration against horseradish peroxidase-conjugated anti-mouse antibody with normal BALB/c mouse serum as the primary antibody. An optimum level of coating was taken as an A_{450} of 1.00. Antibody subtyping was performed in an ELISA (described above).

Absorption experiments. (i) Absorption of MAb with intact *F. nucleatum* 263 cells. A suspension of washed intact *F. nucleatum* 263 cells at an A_{750} of 2.15 was diluted twofold in PBS, and then 40 μl of each dilution of bacteria (neat to 1:32) was added to the wells of a 96-well V-bottom plate (Disposable Products). MAb (120 μl) at a standard dilution was added to all bacterial suspensions. This standard dilution had been adjusted so that it gave an A_{450} of 1.00 when reacted in an ELISA with *F. nucleatum* 263-coated plates. Wells containing diluted MAb only were included as nonabsorbed controls. Plates were incubated for 2 h at RT with occasional agitation and then overnight at 4°C.

The bacteria were pelleted in the microplates by centrifu-

gation at 1,750 × g for 20 min at 4°C, and 100 μl of supernatant was carefully removed from each well and transferred to wells coated with *F. nucleatum* 263. Readings from triplicate wells were averaged and then calculated as a percentage of the reactivity of an unabsorbed MAb to *F. nucleatum* 263, which was taken as 100% binding activity. The residual binding activity was assessed by an ELISA of the absorptions performed in triplicate.

(ii) Absorption of MAb with sonicated *F. nucleatum* 263 cells. A washed suspension of *F. nucleatum* 263 cells was prepared as in section i above. The bacterial suspension (1 ml) was mixed with 100 μl of 20% sodium dodecyl sulfate (Bio-Rad Laboratories, Richmond, Calf.) and 10 μl of β-mercaptoethanol (Sigma), placed in a sonicated water bath (model B12; Branson Sonic Power Co., Danbury, Conn.) until the suspension was visually clear, and then centrifuged for 5 min at 9,000 × g (Beckman Microfuge-B). The protein concentration was equal to 8.0 mg/ml, as determined by the method of Lowry et al. (18). A 10-fold dilution series (10^{-1} to 10^{-5}) of the sonic extract was prepared, and 40 μl of the diluted sonic extract was mixed with 120 μl of diluted MAb (as in section i above) in small serofuge tubes and incubated for 2 h at RT and then overnight at 4°C. The mixture was centrifuged at 9,000 × g (Beckman Microfuge-B) for 5 min, and then the supernatant was treated as described in i above.

Additive binding assay. The method used for the assay of additive binding was described by Friguet et al. (8). Each MAb was titrated in an indirect ELISA against glutaraldehyde-fixed *F. nucleatum* 263 cells to determine the saturation binding level. Then the antibodies were mixed together in a 1:1 ratio in a checkerboard manner, and 100 μl of this mixture was added to *F. nucleatum*-coated 96-well plates. The reactivity was determined by an ELISA after overnight incubation at 4°C.

The additive or nonadditive nature of the binding of the MAbs to *F. nucleatum* 263 was expressed as a percentage calculated by the formula derived by Friguet et al. (8): additive binding = $\{[2A_{1+2}/(A_1 + A_2)] - 1\} \times 100$, where A_{1+2} is the saturation binding of the mixture of MAbs, and A_1 and A_2 are the individual saturation binding levels for each antibody.

Preparation of peroxidase-labeled MAb by using a two-step glutaraldehyde method. MAb 4BA4 was labeled with horseradish peroxidase (Sigma P3875, type VI; 300 U/mg of protein) by the method of Avrameas et al. (1) as described by Voller et al. (33). Briefly, 10 mg of peroxidase in 0.2 ml of 0.1 M phosphate buffer (pH 6.8) containing 1.25% glutaraldehyde was allowed to stand overnight at RT. The glutaraldehyde was removed by passage through a Sephadex G-25 column (PD-10 column; Pharmacia, Uppsala, Sweden) which had been equilibrated with normal saline. The eluate was concentrated to 1 ml by vacuum dialysis (Visking dialysis tubing; molecular weight cutoff, 12,000 to 14,000; Union Carbide Corp.) and added to an equal volume of normal saline containing 5 mg of antibody per ml. The antibody had been prepared from ascitic fluid by precipitation with 45% ammonium sulfate (35), and the antibody concentration was determined by A_{280}. Sodium carbonate-bicarbonate buffer (1 M; pH 9.5; 100 μl) was added to the antibody solution and allowed to stand for 24 h at 4°C, and then 200 μl of 0.1 M lysine monohydrochloride (BDH) was added and, after standing for 2 h at RT, extensively dialyzed against PBS at 4°C. The conjugate was made to 50% concentration with glycerol (pH 7.2) containing 1.0% bovine serum albumin and stored at −20°C as aliquots. The protein/conjugate ratio of 0.35 was determined by A_{280} and A_{490}.

Inhibition of binding of peroxidase-labeled MAb 4BA4 to *F. nucleatum* 263 by unlabeled MAbs. Unlabeled MAbs (100 μl) at the saturation binding level (described above) were added to *F. nucleatum*-coated ELISA 96-well plates, incubated overnight at 4°C, and then washed three times with PBS-T. After nonspecific absorption was blocked with PBS-T–bovine serum albumin, 100 μl of peroxidase-labeled MAb 4BA4 (1:1,000) was added to the wells, and the plates were incubated for 60 min at RT. Inhibition of binding was determined by an ELISA.

RESULTS

Production of hybridomas. After somatic cell hybridization, 156 hybrids were identified from four fusions of immune BALB/c splenocytes with the immune myeloma cells (NSI), and, of these, 20 hybrids produced MAbs that reacted specifically in the screening ELISA against the *F. nucleatum* 263 immunizing strain. A positive control (serum from a BALB/c mouse hyperimmunized against *F. nucleatum*, diluted 1:1,000 in PBS) and a negative control (normal whole BALB/c mouse serum diluted 1:100) were included in the screening assays.

Specificity and reactivity of MAbs to *F. nucleatum* 263. The positive clones were further tested against a panel of bacteria (described in Materials and Methods), and it was found that these 20 MAbs could be further subdivided into four reactivity groups: group 1 (eight MAbs) reacted against all the bacteria tested (data not shown); group 2 (seven MAbs) reacted only with the immunizing strain, *F. nucleatum* 263; group 3 (two MAbs) reacted with *F. nucleatum* 263 and ATCC 25586; and group 4 (three MAbs) reacted against all *F. nucleatum* strains (263, ATCC 25586, and UQD-003) and also cross-reacted with *F. russii* ATCC 25533. No reactivity was found against the other bacterial species tested.

The reactivity results were expressed as a graded ELISA score from 0 to 4 based on optical density at 450 nm as follows: <0.10 = 0, 0.11 to 0.50 = 1, 0.51 to 1.00 = 2, 1.01 to 2.00 = 3, and >2.00 = 4. The readings were recorded as averages for triplicate wells corrected by the subtraction of readings from wells without the primary antibody. The readings were obtained from a 1:10 dilution of tissue culture supernatant in PBS, in which the hybrids were grown to maximal cell density before testing for reactivity. The specificity and graded reactivity of the MAbs and their isotype and subclass determination are shown in Table 1.

Characteristics of selected MAbs. Ten MAbs were selected for further characterization (five MAbs from group 2 and all MAbs from groups 3 and 4) (Table 1). MAbs 4AB5 and 4BC2 were not selected due to their low specific reactivities of 0.22 and 0.27, respectively, in an ELISA, and in addition the hybridomas were difficult to grow in culture. The selected MAbs were cloned by limiting dilution, grown in culture, and passaged through pristane-treated BALB/c mice to produce malignant ascites.

MAb 4BD6 could not be produced as an ascitic fluid and was therefore propagated only in tissue culture. Unfortunately, this hybridoma was eventually lost.

Absorption experiments. (i) Absorption of MAbs with intact *F. nucleatum* 263 cells. MAbs 4BA4, 4BB1, 4AA1, and 7CA4 were used as 45% ammonium sulfate preparations. 7DB5, 7DA6, 7AC5, 7AA3, and 6CD2 were used as raw ascitic fluid (heat inactivated), and 4BD6 was used as a tissue culture supernatant. The residual binding activity of MAbs 4BA4 and 4BD6 was absorbed out completely by a 1:16 dilution of intact *F. nucleatum* 263 cells. MAbs 4BB1 and 4AA1 were

TABLE 1. Specificity and reactivity of MAbs against a bacterial panel

Reactivity group	MAb[a]	Subclass and isotype[b]	ELISA score with[c]: *F. nucleatum* 263	*F. nucleatum* ATCC 25586	*F. nucleatum* UQD-003	*F. russii* ATCC 25533
2	4BA4	IgG2a(κ)	4	0	0	0
	4BB1	IgG2a(κ)	2	0	0	0
	4AA1	IgG1(κ)	2	0	0	0
	4BD6	IgG1(κ)	3	0	0	0
	4AB5	IgM(κ)	1	0	0	0
	7DB5	IgG2b(κ)	2	0	0	0
	4BC2	IgG2b(κ)	1	0	0	0
3	7DA6	IgG2a(κ)	1	2	0	0
	7AC5	IgG1(κ)	1	2	0	0
4	7AA3	IgG1(κ)	4	1	4	1
	6CD2	IgG1(κ)	1	1	1	1
	7CA4	IgG1(κ)	4	2	4	1

[a] Tissue culture supernatants (diluted 1:10 in PBS) harvested from cultures grown to maximal cell densities.
[b] MAb isotype and subclass determined by ELISA.
[c] Results are expressed as a graded ELISA score based on optical density at 450 nm as follows: 0 = <0.10, 1 = 0.11 to 0.50, 2 = 0.51 to 1.00, 3 = 1.01 to 2.00, and 4 = >2.00.

not absorbed out, residual binding levels being 64 and 60%, respectively, when absorbed with the neat suspension of *F. nucleatum* and not greater than 50% at all other dilutions; subsequent testing has produced the same results. This was further investigated using a sonicated preparation of *F. nucleatum* 263 (see below). MAb 7DB5 was not absorbed out and had a residual binding activity of 27 and 31% in the 1:8-to-neat range, rising to 50% at a 1:32 dilution. The results in Fig. 1a illustrate the residual binding of MAbs of reactivity group 2, which are specific for *F. nucleatum* 263 only.

Residual activity remaining after absorption of MAb groups 3 and 4 showed similar patterns (Fig. 1b). After absorption, binding activity for MAbs 7AC5 and 6CD2 was less than 10% over the dilution range, whereas 7CA4 was absorbed out with the neat suspension (2% activity), with activity rising gradually to 53% at a 1:32 dilution. MAb 7AA3 had 35% reactivity remaining after absorption with the neat suspension, which rose to 97% at dilutions of 1:8 to 1:32, and 7DA6 had 45% remaining with the neat suspension, with a gradual rise to 77% at 1:32.

(ii) Absorption of MAbs 4BB1 and 4AA1 with sonicated *F. nucleatum* 263. After absorption with a 1:10 dilution of sonic extract, the residual binding of 4BB1 was absorbed out completely, whereas 52% activity remained for 4AA1. When the concentration of the bacterial sonic extract was decreased to 1:100, 80% residual binding activity remained for both 4BB1 and 4AA1. The activity rose to 91 and 93%, respectively, with a 1:1,000 dilution of sonic extract and 94 and 96%, respectively, with a 1:100,000 dilution. The results are shown in Fig. 2.

(iii) Absorption of MAb by *F. nucleatum* and *F. russii*. MAb tissue culture supernatants were absorbed at their 50% binding level with *F. nucleatum* 263, ATCC 25586, and UQD-003 and *F. russii* ATCC 25533. The supernatants were tested for residual antibody activity in an indirect ELISA against the four bacterial strains coated in 96-well plates and compared with an unabsorbed control. The results obtained with MAbs from group 2, which reacted specifically with *F. nucleatum* 263 (Table 1), showed that MAbs 4BA4 and 4BD6 reacted with and were absorbed out completely by *F. nucleatum* 263. MAbs 4BB1 and 4AA1 reacted with but were not absorbed out by intact *F. nucleatum* 263 cells. This group of MAbs did not react with and were not absorbed out by *F. nucleatum* ATCC 25586 or UQD-003 or *F. russii* ATCC 25533 (Table 2).

In group 3 (7DA6 and 7AC5), both MAbs reacted with *F. nucleatum* 263 and ATCC 25586, but when MAb 7AC5 was absorbed with intact *F. nucleatum* 263 cells, this resulted in a residual binding activity of 82%, whereas the residual binding activity remaining after absorption with *F. nucleatum* ATCC 25586 was 37%. When MAb 7DA6 was absorbed with *F. nucleatum* 263 and ATCC 25586, the binding activity remaining was 92 and 77%, respectively (Table 2).

In group 4, MAbs 7AA3, 6CD2, and 7AC4 reacted with *F. nucleatum* 263, ATCC 25586, and UQD-003 and also with *F. russii* ATCC 25533. MAb 7AA3 reacted strongly with *F. nucleatum* 263 and UQD-003, moderately with *F. nucleatum*

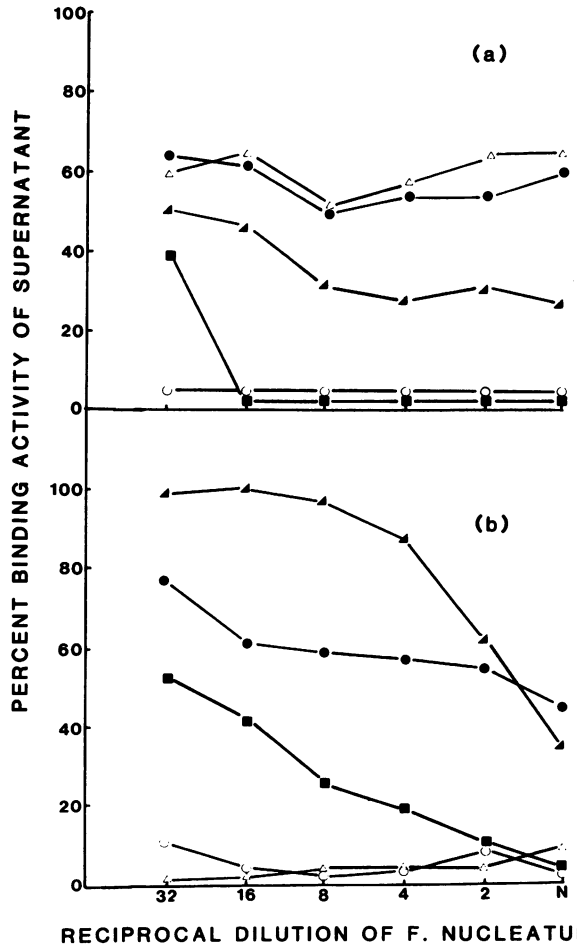

FIG. 1. Residual binding activity of supernatants determined after absorption with intact *F. nucleatum* 263 cells. The reactivity was calculated as a percentage of the reactivity of an unabsorbed MAb to *F. nucleatum* 263, which was taken as 100% binding activity. A twofold dilution series of bacteria was mixed with MAbs at a dilution adjusted to give an A_{450} of 1.00 in an ELISA with *F. nucleatum* 263-glutaraldehyde-coated plates. Results are shown for the following MAbs at the indicated dilutions. (a) ○, 4BA4 (1:20,000); △, 4BB1 (1:40); ●, 4AA1 (1:100); ■, 4BD6 (1:100); ▲, 7DB5 (1:80). (b) ○, 6CD2 (1:300); ▲, 7AA3 (1:5,000); ■, 7CA4 (1:2,000); ●, 7DA6 (1:200); △, 7AC5 (1:150).

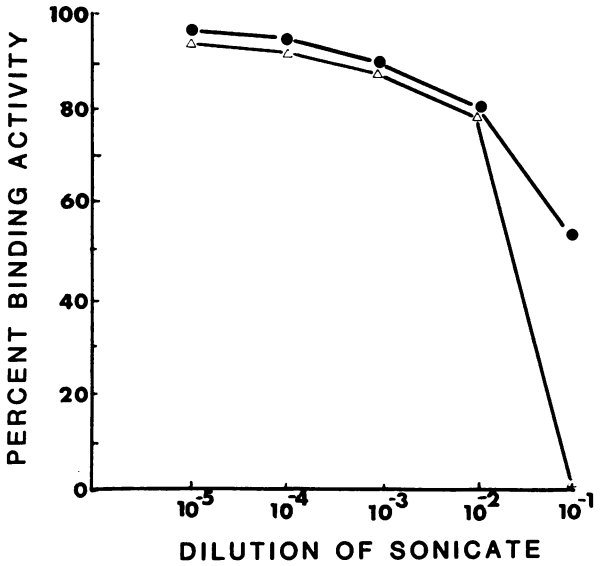

FIG. 2. Residual binding activity of supernatants determined after absorption with sonicated *F. nucleatum* 263 cells. The reactivity was calculated as a percentage of the reactivity of an unabsorbed MAb to *F. nucleatum* 263, which was taken as 100% binding activity. The sonic extract (protein concentration, 8 mg/ml) was diluted in a 10-fold series and mixed with MAbs adjusted to a dilution that gave an A_{450} of 1.00 in an ELISA with *F. nucleatum*-glutaraldehyde-coated plates. Shown are results for MAb 4AA1 at a 1:40 dilution (●) and MAb 4BB1 at a 1:100 dilution (△).

ATCC 25586, and weakly with *F. russii* ATCC 25533. In aborption studies with *F. nucleatum* strains, the remaining residual activity of the supernatant was *F. nucleatum* 263, 13%; *F. nucleatum* ATCC 25586, 18%; *F. nucleatum* UQD-003, 20%; and *F. russii*, 67%. MAb 6CD2 reacted less with *F. nucleatum* ATCC 25586 than with the other three bacterial strains. Absorption with the bacteria gave residual levels as follows: *F. nucleatum* 263, 60%; *F. nucleatum* ATCC 25586, 56%; *F. nucleatum* UQD-003, 52%; and *F. russii*, 40%. MAb 7CA4 reacted strongly with *F. nucleatum* 263 and UQD-003, moderately with *F. nucleatum* ATCC 25586, and weakly with *F. russii*. When 7CA4 was absorbed with the bacterial strains, the residual levels were as follows: *F. nucleatum* 263, 22%; *F. nucleatum* ATCC 25586, 17%; *F. nucleatum* UQD-003, 16%; and *F. russii*, 64%. The results are shown in Table 2.

TABLE 2. Comparison of MAb reactivity before and after absorption with each strain of *F. nucleatum* and *F. russii* ATCC 25533[a]

MAb	Dilution[b]	Optical density at 450 nm with[c]:			
		F. nucleatum			*F. russii* ATCC 25533
		263	ATCC 25586	UQD-003	
4BA4	1:100	0.22/1.40	0.18/0.16	0.19/0.20	0.16/0.18
4BD6	1:100	0.41/1.00	0.13/0.17	0.20/0.22	0.17/0.17
4BB1	1:10	0.33/0.28	0.16/0.15	0.18/0.19	0.18/0.16
4AA1	1:10	0.34/0.33	0.13/0.16	0.20/0.18	0.16/0.15
7DA6	1:10	0.34/0.37	0.30/0.39	0.15/0.14	0.16/0.18
7AC5	1:10	0.23/0.28	0.16/0.43	0.20/0.19	0.17/0.17
7AA3	1:100	0.24/1.90	0.18/1.00	0.34/1.70	0.18/0.26
6CD2	1:100	0.27/0.44	0.13/0.23	0.22/0.42	0.20/0.49
7CA4	1:100	0.42/1.92	0.16/0.93	0.30/1.86	0.18/0.28

[a] Supernatants were reacted in an ELISA in which plates were coated with the absorbent bacterial strain.
[b] Dilution of tissue culture supernatant giving 50% binding to glutaraldehyde-bacteria-coated 96-well plates, determined by titration.
[c] Absorbed MAb/unabsorbed MAb. Readings of triplicate results corrected for blank readings. Bacterial suspensions were used at an A_{750} of 0.50.

TABLE 3. Additive binding assay to determine additive or nonadditive nature of reactivity of MAbs to *F. nucleatum* 263-coated ELISA plates

MAb[a]	Additive binding index (%)[b] with MAb:								
	4BB1	4AA1	4BD6	7DB5	7DA6	7AC5	7AA3	6CD2	7CA4
4BA4	39	55	34	62	50	58	61	71	47
4BB1		27	28	20	20	33	90	94	76
4AA1			4	45	63	25	62	38	48
4BD6				33	27	16	30	4	25
7DB5					45	13	106	48	101
7DA6						31	95	51	81
7AC5							11	13	24
7AA3								76	17
6CD2									72

[a] MAbs diluted and used at saturated binding levels.
[b] Additive binding index calculated by formula derived by Friguet et al. (8) (see the text).

Additive binding assay. The results which indicate whether the MAbs reacted with the same or different epitopes on *F. nucleatum* 263 are shown in Table 3. The absence of nonspecific binding was determined by the reactivity of the MAbs at the saturated binding levels against *F. mortiferum* ATCC 25551 and *B. gingivalis* ATCC 33277.

Inhibition of binding of peroxidase-labeled 4BA4 by unlabeled MAbs. An experiment was performed to determine the inhibition of binding of peroxidase-labeled MAb 4BA4 by unlabeled MAbs. The MAbs were used at their saturation binding capacity. Horseradish peroxidase-conjugated 4BA4 was found to be inhibited by unlabeled 4BA4. No inhibition of binding of conjugated 4BA4 was observed with the other unlabeled MAbs.

DISCUSSION

In this paper we describe the production of hybrid cell lines that produce MAbs directed against antigens of *F. nucleatum* 263 and report on the cross-reactivity of these MAbs with other strains of *F. nucleatum* (ATCC 25586 and UQD-003) and another *Fusobacterium* species, *F. russii* ATCC 25533. All other *Fusobacterium* species and bacterial genera that were tested, such as *Bacteroides*, *Actinomyces*, *Streptococcus*, and *Escherichia*, were negative.

Initial analysis of the reactivity and specificity of these MAbs used whole bacteria in an indirect ELISA under conditions of antibody excess. The results were then extended in absorption studies with suspensions of whole bacteria, and when low levels of absorption were detected, sonicated bacteria were used. In the latter method of testing, the MAbs were used in a quantitative absorption assay for the expression and recognition of antigens under conditions of antigen excess. With these approaches, the differences in reactivity were a reflection not only of the number of antibody molecules present but also of the affinity and avidity of the antibody, the epitope density, and the accessibility of the target antigen.

The reactivity of one group of MAbs (group 2) was with a strain-specific antigen (or antigens) which was only detected on *F. nucleatum* 263. MAbs in group 3 reacted with an antigen that was shared by both *F. nucleatum* strains, 263 and ATCC 25586.

A group-specific antigen was detected on the three *F. nucleatum* strains (263, ATCC 25586, and UQD-003) by another group of MAbs (group 4), and this antigen (or antigens) was also detected on another *Fusobacterium* species, *F. russii* ATCC 25533. All the bacteria used in this study were grown under the same cultural conditions, and therefore the presence of antigenic determinants on these bacteria related only to species or strain differences.

It has been shown that there are different electrophoretic protein patterns among different strains of *F. nucleatum* (20); morphological and physiological differences also exist among the strains (30). In a recent study it was found that there was genetic heterogeneity among 58 strains isolated (Y. Selin and J. L. Johnson, J. Dent. Res. **60**[Special Issue A]:415, abstr. no. 420, 1981). This suggests that antigenic differences may be possible within the *F. nucleatum* strains. Other investigators have reported cross-reactivity for polyclonal antiserum produced in rabbits (6). These workers showed that rabbit antisera to *F. nucleatum* ATCC 10953 and ATCC 23726 reacted with both *F. nucleatum* strains but showed no cross-reactivity with *F. mortiferum* or *F. varium*. In a later study, Falkler et al. (7) produced rabbit anti-*F. nucleatum* ATCC 10953 serum which cross-reacted with three strains of *F. nucleatum* (ATCC 10953, ATCC 4355, and ATCC 10195). Stauffer et al. (29) demonstrated that another rabbit antiserum against fusobacteria detected strain specificity, specificity for groups within subspecies, occasionally specificity among strains representing subspecies. They suggested that a study of additional strains might demonstrate the existence of distinct antigenic groups within the species. Even though there is heterogeneity among the strains of *F. nucleatum*, it has been reported that the major antigenic determinants of the different isolates are similar (32). Indeed, in a report of a DNA-DNA hybridization study of 16 strains of *F. nucleatum* (24), it was concluded that *F. nucleatum* comprises a heterogeneous group of microorganisms closely related to *Fusobacterium periodonticum* and *Fusobacterium simiae* but unrelated to other *Fusobacterium* species tested. This study did not include *F. russii*. MAbs have been generated that cross-react with *F. nucleatum* strains and *F. periodonticum*, showing a close relationship between these species (3).

In the present report, species-specific, strain-specific, and cross-reactive MAbs are described. It would be of interest to define a pattern of antigenic relationship, using these MAbs, within a larger number of *F. nucleatum* strains. MAbs then could be used as diagnostic reagents in the identification of *F. nucleatum* isolates. Species-specific antisera have been used in an indirect immunofluorescence assay for the rapid detection and identification of *Fusobacterium* species in clinical specimens (29). Similarly, the MAbs described in the present report have been used to detect *F. nucleatum* in dental plaque. Further work will be necessary to determine whether such an approach has clinical applications.

In the present study, whole "aerobically" killed bacteria were used as immunogens, and in the screening ELISA, glutaraldehyde-fixed whole bacteria were used. The reactivity with whole bacteria in absorption experiments showed that the antigenic character was unaltered by these treatments. Nevertheless, it has been reported that Formalin fixation denatures the antigens and that these antigens may not be detected in an ELISA (2).

The relationship of the MAbs to the epitopes on *F. nucleatum* 263 was determined by the additive binding assay (8). The results are expressed as percentages, with lower percentages suggesting epitope sharing. The percentages obtained therefore indicate whether the MAbs could be sharing the same epitope or whether different epitopes may be involved; they may also indicate the degree of overlap between epitopes. There was an indication that MAbs 4BD6 and 4AA1 (4%) compete for the same epitope; similarly, 4BD6 and 6CD2 (4%) may share an epitope. The same antigenic determinant could be detected by the MAbs reacting with different antigenic sites on the antigen which may be partially shared by the MAbs or could be epitopes at some distance from one another. The range of percentages shown in Table 3 suggests the different degrees of overlap that may exist between the epitopes detected. Some variability in the results was noted in the additive binding assay, and it was thought that a better approach to the study of the relationship of MAbs to epitope binding would be a competitive inhibition assay with labeled and unlabeled MAbs. In such an assay, horseradish peroxidase-conjugated MAb 4BA4 was shown not to be inhibited by any unlabeled MAb other than 4BA4. Knowing whether MAbs react with the same or different epitopes helps in developing a better set of reagents for use in diagnosis and in immunological probes.

The nature of the antigens recognized by the MAbs is unknown and subject to current investigations. The MAbs may react with protein, lipopolysaccharide, or lipid components of the bacterial cell wall. The main purpose of this study was the production of the MAbs to enable the isolation and purification of bacterial antigens which could then be used to elicit an immunological response of known specificity.

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