INFECTION AND IMMUNITY, Jan. 1988, p. 219–224
0019-9567/88/010219-06$02.00/0

Vol. 56, No. 1

Characterization of Monoclonal Antibodies to Fimbria-Associated Adhesins of *Bacteroides loescheii* PK1295†

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Received 16 July 1987/Accepted 6 October 1987

***Bacteroides loescheii* PK1295 fimbriae, which mediate the lactose-sensitive coaggregation with *Streptococcus sanguis* 34 and the lactose-insensitive coaggregation with *Actinomyces israelii* PK14, were injected into mice to raise adhesin-specific monoclonal antibodies (MAbs). Supernatants of hybridomas were screened for the capacity to inhibit coaggregation and agglutinate intact bacteria. Of the 10 MAbs that were isolated, 4 were specific and potent inhibitors of the coaggregation between *B. loescheii* and *S. sanguis* and two other MAbs specifically inhibited the *B. loescheii-A. israelii* interaction. None of the six MAbs which inhibited adherence were capable of agglutinating whole cells of *B. loescheii*, whereas the four remaining MAbs agglutinated whole cells but had no effect on coaggregation. Fab fragments of two MAbs, one that inhibited the coaggregation with *S. sanguis* and another that inhibited the interaction with *A. israelii*, also were shown to inhibit the respective coaggregation interactions, suggesting that each of the immunoglobulins recognized its adhesin molecule at or near the active sites. By immunoblotting or immunoprecipitation, the *S. sanguis* adhesin-specific MAbs reacted with a 75-kilodalton polypeptide present in fimbria-enriched preparations, whereas the *A. israelii* adhesin-specific MAbs recognized a 45-kilodalton polypeptide in the same preparations. By screening hybridoma supernatants directly for their capacity to block coaggregation, we isolated MAbs which were used to establish that the *B. loescheii-S. sanguis* and the *B. loescheii-A. israelii* interactions were mediated by different adhesins.**

Interbacterial adherence is thought to be involved in the colonization and formation of dental plaque. Coaggregation is a specific bacterial adherence interaction observed among a variety of oral microorganisms from different species and genera (2, 6, 9, 10). The gram-negative oral bacterium *Bacteroides loescheii* PK1295 forms multigeneric aggregates by bridging between otherwise noncoaggregating gram-positive microorganisms such as *Streptococcus sanguis* 34 and *Actinomyces israelii* PK14 (9). The coaggregation with *S. sanguis* is inhibited by lactose, whereas the interaction with *A. israelii* is not affected by more than 80 carbohydrate compounds tested (15). Recently, we tentatively identified the adhesins on the surface of *B. loescheii* that mediate the interactions with its two partner strains (15). Mutants lacking both adhesin activities were used to adsorb rabbit antisera prepared against *B. loescheii*. The adsorbed sera did not agglutinate *B. loescheii* but effectively blocked coaggregation with both partners. Immunoblots developed with the adsorbed sera showed 75- and 45-kilodalton (kDa) polypeptides associated with the purified fimbriae from wild-type cells. Fimbria-containing preparations from mutants unable to coaggregate with *S. sanguis* were missing the 75-kDa polypeptide but retained the 45-kDa polypeptide. The mutants that did not coaggregate with both partners lacked both polypeptides. By inference then, the 75- and the 45-kDa polypeptides were associated with the adhesins mediating coaggregation with *S. sanguis* and *A. israelii*, respectively.

The purpose of the present study was to identify the surface adhesins that mediate coaggregation. Since purified *B. loescheii* fimbriae aggregated *S. sanguis* and *A. israelii* cells, suspensions of these organelles were used to immunize mice to produce monoclonal antibodies (MAbs). In this report we describe the screening strategy used to select adhesin-specific (coaggregation-inhibitory) MAbs and their properties. These MAbs were then used to identify the fimbria-associated proteins responsible for the *B. loescheii-S. sanguis* and *B. loescheii-A. israelii* coaggregations. We also report the isolation and characterization of MAbs with other specificities.

MATERIALS AND METHODS

Bacterial strains and antigens. The bacterial strains used in this study (*B. loescheii* PK1295, *S. sanguis* 34, and *A. israelii* PK14) were of human dental plaque origin; optimal conditions for their growth have been described elsewhere (9). Bacterial cells were harvested and washed twice in coaggregation buffer (0.15 M NaCl, 10^{-4} M $CaCl_2$, 10^{-4} M $MgCl_2$, 0.02% NaN_3, and 10^{-3} M Tris hydrochloride [pH 7.4]). Cells were stored as pellets at −20°C or suspended in coaggregation buffer and maintained at 4°C. The isolation of *B. loescheii* fimbriae was described previously (15). Briefly, cells were made up to 10% suspension (wet wt/vol) in coaggregation buffer and exposed to mild sonic oscillation (Mini Sonifier; Kontes, Vineland, N.J.). Whole cells and large membrane particles were removed by centrifugation and filtration. Concentrated supernatants were treated with 0.25% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) (Sigma Chemical Co., St. Louis, Mo.) and applied to a Sephacryl S-500 molecular sieving column (Pharmacia, Inc., Piscataway, N.J.) equilibrated with coaggregation buffer containing 0.25% CHAPS. The fimbriae that were eluted in the void volume agglutinated both *S. sanguis* and *A. israelii* whole cells.

Preparation of hybridomas. Ten 8-week-old BALB/c female mice were immunized with *B. loescheii* fimbriae. On day 0, each animal received 40 μg of fimbrial protein in

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† This study is dedicated to our friend and colleague, Charles Louis Wittenberger, who died unexpectedly on 27 June 1987.

complete Freund adjuvant administered in the footpads. Every 21 days thereafter, the mice received subcutaneous booster injections containing 30 μg of antigen protein in Freund adjuvant. One week after administration of the fifth booster, the sera from the animals were tested for inhibition of coaggregation and for bacterial agglutination. The spleen cells from the mouse producing the highest titer of antibody were transferred into six irradiated syngeneic recipients (5). Immediately after the adoptive transfer, the animals received 5 μg of antigen injected intraperitoneally. Six days after the adoptive transfer, the spleen cells were fused with the X63-Ag8-653 myeloma cell line (7). The resulting hybridoma supernatants were screened at 2 and 3 weeks for the capacity to inhibit coaggregation and to agglutinate bacteria. The positive cultures were expanded, retested, and cloned by limiting dilution on thymocyte feeder layers (5). The lines obtained were finally injected into irradiated (250 rads) BALB/c mice previously primed with 0.5 ml of pristane.

Screening of hybridoma supernatants. Coaggregation-blocking antibody was detected by adding 50 μl of *B. loescheii* cell suspension adjusted to a value of 260 Klett units (ca. 5×10^9 cells per ml [red filter, Klett-Summerson colorimeter; Klett Mfg. Co., Long Island City, N.Y.]) to glass test tubes (10 by 75 mm) with 50 μl of hybridoma culture supernatant and incubated for 30 min. After the addition of 50 μl of *S. sanguis* or *A. israelii* (5×10^9 cells per ml), the contents of the test tubes were mixed and immediately scored for visible coaggregation (2). Agglutination assays of intact *B. loescheii* cells were performed in capillary tubes (20 μl volume) by mixing equal volumes of bacteroides cells (260 Klett units) and culture supernatant diluted 1:1, 1:10, or 1:100 in coaggregation buffer. The extent of agglutination was determined visually after 1 h and 24 h. When agglutinating antibody was tested for inhibition of coaggregation by preincubating hybridoma supernatants with *B. loescheii* cells, a slight background caused by agglutination was observed after 30 min of incubation. However, this did not affect evaluation of the reaction, because the aggregates were dispersed after mixing and did not reform during the coaggregation assay.

Purification of MAbs and Fab fragments. Ascites fluid was collected and immunoglobulin was purified by $(NH_4)_2SO_4$ precipitation (0 to 55% fraction) and ion-exchange chromatography on cellulose (DE-52; Whatman, Inc., Clifton, N.J.). The purity of each monoclonal antibody was evaluated by polyacrylamide gel electrophoresis (8). Isotypes were determined by immunodiffusion.

Fab fragments were prepared by treating purified MAb with papain in 0.2 M sodium acetate buffer containing 4 mM EDTA and 4 mM cysteine, pH 5.8, for 4 h at 37°C. Fab fragments were purified by ion-exchange chromatography (DEAE-cellulose) and gel filtration on Sephadex G-75 (Pharmacia Fine Chemicals, Piscataway, N.J.). Purity of the Fab preparations was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Quantitative assay for inhibition of coaggregation. The inhibition of coaggregation by the appropriate MAbs and Fab fragments was quantitated by a spectrophotometric assay similar to that originally described by McIntire et al. (10). In these assays, twofold dilutions of the respective MAb or Fab fragments were added to test tubes containing 200 μl of a *B. loescheii* cell suspension (260 Klett units) and the tubes were incubated at room temperature for 30 min. Two hundred microliters of the appropriate coaggregation partner was added to each tube, mixed vigorously for 10 s, and centrifuged at 600 × *g* for 1 min. Supernatants were carefully removed, and the optical density at 600 nm was determined with a model 2400S recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Controls consisted of *B. loescheii* cell suspensions, the respective coaggregation partners, and normal mouse immunoglobulin G (IgG). The inhibition was expressed as the percent decrease in coaggregation relative to the coaggregation in the presence of normal mouse IgG or coaggregation buffer. All experimental points were determined in triplicate.

Immunological methods. Whole cells of *B. loescheii* were suspended in 0.2 M sodium carbonate buffer, pH 9.6, and were adsorbed onto 96-well microtiter plates as solid-phase antigens for enzyme-linked immunosorbent assays (ELISAs). Purified MAb IgG was added in the range of 0.1 ng to 100 μg. After 1 h, biotinylated sheep anti-mouse IgG was added to each well, followed by streptavidin-biotinylated horseradish peroxidase complex according to the instructions of the manufacturer (Amersham Corp., Arlington Heights, Ill.). Chromophore generation was measured with the Titertek Multiskan MC (Flow Laboratories, Inc., McLean, Va.).

For immunoblots, ultrasonically disrupted preparations of *B. loescheii* surface components were separated on preparative SDS gels (8). The polypeptides resolved on gels were transferred onto nitrocellulose membranes (1, 13), which were cut into 1.5-mm-wide strips for screening positive hybridoma cultures and purified MAb IgG. Membrane strips were treated with the primary antibody (MAb) and reacted with biotinylated anti-mouse IgG followed by streptavidin-biotinylated horseradish peroxidase according to the instructions of the manufacturer. Immunoreactive bands were visualized with hydrogen peroxide and 4-chloro-1-naphthol (Bio-Rad Laboratories, Richmond, Calif.).

A MAb IgG affinity matrix was prepared by reacting MAb 5BB1-2 with cyanogen bromide-activated Sepharose 4B beads according to the instructions of the manufacturer (Pharmacia Fine Chemicals). Three milliliters of matrix beads to which 15 mg of MAb protein was coupled was incubated with a high-speed supernatant (250,000 × *g* for 2 h) of an ultrasonically treated preparation (model 350 sonifier; Heat Systems-Ultrasonics, Inc., Farmingdale, N.Y.) of *B. loescheii* cells. The incubation was performed in the presence of 10 mM EDTA and 5 mM phenylmethylsulfonyl fluoride for 90 min at room temperature with continuous mixing. After successive washes with 1 M KCl–0.25% CHAPS and six washes with phosphate-buffered saline containing EDTA and phenylmethylsulfonyl fluoride, the matrix was eluted with a solution of 2% acetic acid. The eluate was concentrated by lyophilization and reconstituted with phosphate-buffered saline. High-speed supernatants (50 μg of protein) and MAb affinity column eluate (1 μg) were applied to anionic slab gels and were electrophoresed (3). The resolved proteins were transferred to nitrocellulose filters and developed as described above by using 5BB1-2 and 3AD6 MAbs as probes.

^{125}I labeling of adhesin and fimbrial components. Concentrated culture supernatants were freed of cells by filtration through Millex GV 0.22-μm-pore-size filters (Millipore Corp., Bedford, Mass.), assayed for the ability to agglutinate cells of *S. sanguis* and *A. israelii*, and subjected to mild sonication (Mini Sonifier; Kontes). This source of adhesin was preferred over ultrasonically disrupted cell suspensions because the latter contained high levels of a protease activity that was refractile to all inhibitors tested and degraded IgG rapidly. One milliliter of this culture supernatant containing 1 to 2 mg of protein was treated with 1 to 1.5 mCi of ^{125}I

(IMS-300; Amersham) in the presence of Iodobeads (Pierce Chemical Co., Rockford, Ill.) according to the instructions of the manufacturer. Unreacted ^{125}I was separated from radiolabeled proteins by passing the mixture over an AG1-X8 Econo-column (Bio-Rad). Specific radioactivity was determined by trichloroacetic acid precipitation after diluting the radiolabeled preparation 1:1,000 in 0.2 M borate buffer, pH 8.0, containing 0.15 M NaCl (BBS) and 2% bovine serum albumin.

Isolation and identification of radiolabeled adhesins. A series of Eppendorf tubes received 60 μg of protein of a ^{125}I-labeled culture supernatant (specific radioactivity, 5.8 μCi/mg of protein), 25 μg of normal mouse IgG, 5 μmol of phenylmethylsulfonyl fluoride, 10 μmol of EDTA, and sufficient goat anti-mouse IgG coupled to Sepharose 4B to bind 50 μg of mouse IgG (Cooper Biomedical, Inc., West Chester, Pa.). All reagents were prepared in BBS, and the volume of each tube was adjusted to 200 μl in the same buffer. This initial reaction mixture served to remove radiolabeled proteins that nonspecifically adsorbed to either the IgG- or anti-IgG-coupled beads. The tubes were incubated for 1 h at 4°C with constant mixing and centrifuged at 13,000 × *g* for 3 min. Supernatants were transferred to fresh tubes, and the beads were washed with 100 μl of BBS; the wash was combined with the supernatant fluid. Each tube containing the supernatants received 25 μl of one of the MAbs (1 μg of protein per μl) and CHAPS to a final concentration of 0.2%. After incubation for 1 h in the cold with mixing, 50 μl of anti-mouse IgG coupled to Sepharose 4B was added to each tube and incubated as before. The beads were centrifuged and washed five times with BBS, and the radioactivity was measured with a Gamma 9000 counter (Beckman Instruments, Inc., Fullerton, Calif.). Controls containing normal mouse IgG and anti-mouse IgG coupled to Sepharose 4B were treated in the same fashion; the counts bound were less than 5% of those bound in the experimental samples. The bead preparations were suspended in 100 μl of a double-strength solution of SDS sample buffer (14) containing 10 mM 2-mercaptoethanol and boiled for 15 min. Boiled supernatants containing at least 10,000 cpm were subjected to slab SDS-PAGE (8). Gels were fixed in 12% trichloroacetic acid for 2 h, washed in 7% acetic acid overnight, dried between sheets of cellophane, and placed on XAR-5 X-ray film (Eastman Kodak Co., Rochester, N.Y.) for 6 to 12 h. Autoradiographs were developed, and the R_fs of the radioactive proteins were determined by comparing the migration rates with those of standard proteins (Bio-Rad) run simultaneously with the ^{125}I-labeled samples.

Protein was determined by the method of Schacterle and Pollack (11) or by using the Bio-Rad protein assay kit.

RESULTS

Screening hybridoma supernatants for the ability to block coaggregation between *B. loescheii* and *S. sanguis* or *A. israelii* and agglutinate intact *B. loescheii* cells yielded a total of 50 positive cultures of 384 tested. Of these 50 hybridomas, 19 inhibited coaggregation between *B. loescheii* and *S. sanguis*, 11 inhibited the *B. loescheii-A. israelii* interaction, and 20 agglutinated whole cells of *B. loescheii*. The cultures with the highest titers for either agglutination or inhibition of coaggregation were expanded and cloned; 10 of these MAb-secreting cell lines were selected for ascites fluid production in mice. The purified immunoglobulins were used in all subsequent experiments.

The isotypes of the 10 MAbs are listed in Table 1. Four MAbs (MAbs 3AD6, 5DB5, 5BB1-2, and 3BD2) inhibited the coaggregation of *B. loescheii* and *S. sanguis*, two other MAbs (2AA1 and 4AC3) inhibited the coaggregation with *A. israelii*, and the remaining four MAbs (5AB6, 5BB1-5, 2BD3, and 5AB5) agglutinated *B. loescheii* cells. No functional cross-reactivities were observed among the three specificity groups (as defined above); for example, the MAbs that inhibited the *B. loescheii-S. sanguis* interaction had no effect on *B. loescheii-A. israelii* coaggregation and did not agglutinate intact cells of *B. loescheii*. Conversely, a MAb that blocked coaggregation between *B. loescheii* and *A. israelii* had no effect on the interaction with *S. sanguis*, nor did it agglutinate cells (Fig. 1). However, when nonagglutinating anti-adhesin MAbs were incubated with *B. loescheii* cells for 30 min and then mixed with anti-mouse IgG, a rapid agglutination of the mixture occurred (data not shown). Immunodiffusion experiments revealed that only agglutinating MAbs formed a precipitin line when reacted with a soluble preparation of cell surface components (data not shown), whereas none of the MAbs that inhibited coaggregation formed insoluble antigen-antibody complexes.

The titers of several of the MAbs were determined by ELISA, and the concentration of antibody required to produce one-half of the maximum antigen-antibody interaction was roughly similar for all IgG preparations tested, with the exception of MAb 4AC3, which was 10-fold higher (Table 1). Substantive differences were observed in the rate and final intensity of color development among the MAbs tested. The agglutinating MAbs, 2BD3, 5BB1-5, and 5AB5, caused an immediate release of chromophore in reactions that exceeded an A_{490} of 2.0, whereas all of the coaggregation-blocking MAbs exhibited a significantly slower rate of color development and a maximal A_{490} of 1.2 or less. The rate of color development was not dependent on the concentration of MAb added to the wells.

When used in immunoblot analyses, two of the MAbs that blocked coaggregation between *B. loescheii* and *S. sanguis*, 5DB5 and 3AD6, reacted with a 75-kDa polypeptide (Fig. 2, lanes 1 and 2). The two other MAbs which inhibited this

TABLE 1. Characterization of anti-*B. loescheii* MAbs

MAb	Isotype	Coaggregation scores with[a]:		Agglutination[b]	ELISA[c]
		S. sanguis 34	*A. israelii* PK14		
3AD6	IgG1	0	4		0.24 (1.2)
5DB5	IgG1	0	4		0.24 (0.9)
5BB1-2	IgG2b	0	4		0.03 (1.2)
3BD2	IgG1	0	4		ND
2AA1	IgG1	4	0		ND
4AC3	IgG2b	4	0		2.0 (0.7)
5AB6	IgG1	4	4	0.06	ND
5BB1-5	IgG1	4	4	0.88	0.12 (>2.0)
2BD3	IgG1	4	4	0.06	0.06 (>2.0)
5AB5	IgG3	4	4	0.24	0.24 (>2.0)

[a] *B. loescheii* cell suspension was preincubated for 30 min with the respective MAb (concentration range, 0.5 to 5 μg of protein per ml), followed by the addition of the coaggregation partner. Coaggregation was scored visually 30 to 40 s after mixing; 0, no coaggregation; 4, maximal coaggregation.

[b] Lowest concentration of MAb (in micrograms of protein per milliliter) producing agglutination in capillary tube assay.

[c] Concentration of MAb (in micrograms of protein per milliliter) producing 50% of maximum color development in ELISA. Values in parentheses are maximum color development attained with each MAb at A_{490}. ND, Not done.

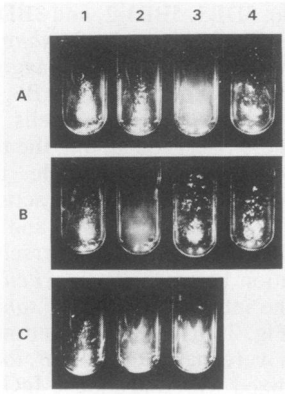

FIG. 1. Visual coaggregation assay used for screening the hybridoma culture supernatant. Row A shows test tubes that received *B. loescheii*, *S. sanguis* 34 cells, and MAb. Row B shows test tubes containing *B. loescheii*, *A. israelii* PK14 cells, and MAb. No coaggregation partner cells were added to tubes in row C. The following hybridoma supernatants were added to test tubes in columns 1 to 3: 2BD3, 4AC3, and 3AD6, respectively. Preimmune serum was added to tubes in column 4.

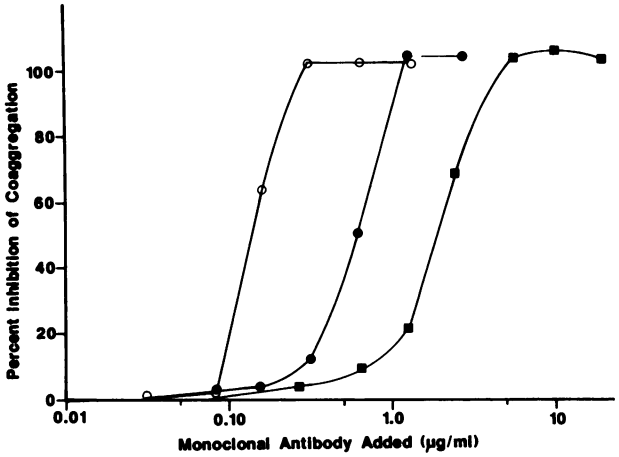

FIG. 3. Inhibition of coaggregation between *B. loescheii* PK1295 and *S. sanguis* 34 by MAb or Fab fragments. Symbols: ○, 5BB1-2 Fab; ●, 5BB1-2 MAb; ■, 5DB5 MAb. Neither normal mouse IgG nor MAb 4AC3 (the *A. israelii*-specific adhesin) showed any inhibition at the maximum IgG concentration tested (10 μg of protein per ml).

interaction, 5BB1-2 and 3BD2, failed to react with any band on the immunoblot transfer. Like the latter two MAbs, the two MAbs that specifically inhibited the *B. loescheii-A. israelii* coaggregation, 4AC3 and 2AA1, also failed to recognize any of the polypeptides on the nitrocellulose membrane. However, of the four agglutinating clones, three (5BB1-5, 2BD3, and 5AB5) reacted with materials of higher molecular mass (ca. 100 to 115 kDa; Fig. 2, lanes 8 to 10).

The inhibitory effects of certain MAbs and Fab fragments on the coaggregation of *B. loescheii* and its two partners were quantitated turbidimetrically. Fifty percent inhibition of the *B. loescheii-S. sanguis* interaction was achieved with 0.68 μg of 5BB1-2 MAb protein per ml (Fig. 3). A higher concentration of 5DB5 (ca. 2.5 μg of protein per ml) was needed for 50% inhibition of the same coaggregation. Fab fragments of 5BB1-2 completely inhibited the interaction, and 50% inhibition was observed at a sixfold lower concentration than that of the intact IgG molecule (0.12 μg of Fab protein per ml). When 4AC3 was used to inhibit the coaggregation between *B. loescheii* and *A. israelii*, 1.1 μg of MAb protein produced a 50% inhibition (Fig. 4). A higher concentration of the 4AC3 Fab, 2.0 μg of protein per ml, was required to inhibit 50% of the interaction.

To identify the proteins that react with each of the coaggregation-inhibiting MAbs, radioiodinated culture supernatants were immunoprecipitated with these antibodies.

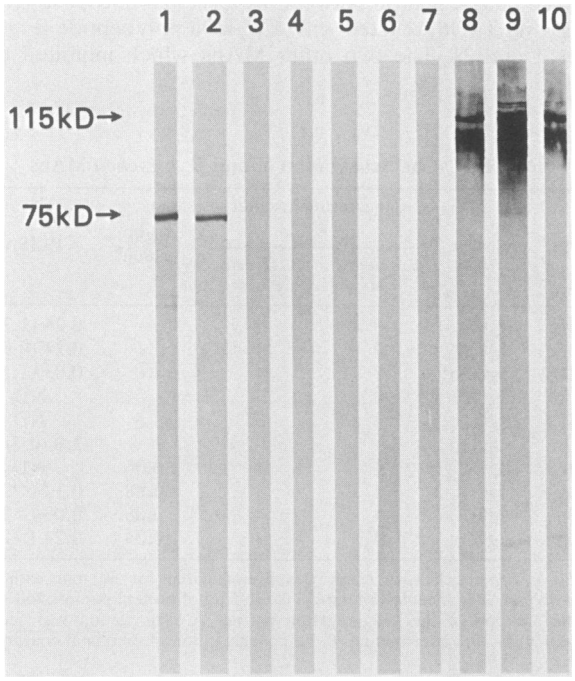

FIG. 2. Immunoblot analysis of *B. loescheii* surface components by MAbs. Lanes 1 through 10 were developed with MAbs 3AD6, 5DB5, 5BB1-2, 3BD2, 4AC3, 2AA1, 5AB6, 5BB1-5, 2BD3, and 5AB5, respectively. kD, Kilodaltons.

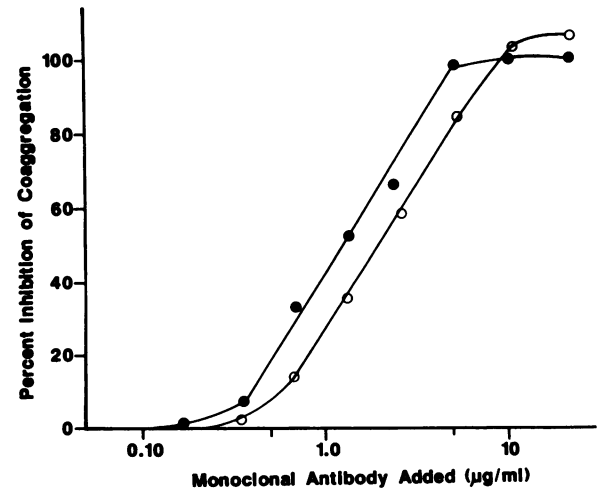

FIG. 4. Inhibition of coaggregation between *B. loescheii* PK1295 and *A. israelii* PK14 by MAb or Fab fragments. Symbols: ○, 4AC3 Fab; ●, 4AC3 MAb. Neither normal mouse IgG nor MAb 5BB1-2 (the *S. sanguis*-specific adhesin) showed any inhibition at the maximum IgG concentration tested (10 μg of protein per ml).

The respective immune complexes were reacted with anti-mouse IgG coupled to Sepharose 4B beads; the beads were washed and treated with SDS and 2-mercaptoethanol, and the constituent proteins were resolved by denaturing PAGE. Results obtained with five of the MAbs are shown in Fig. 5. A 75-kDa radioactive polypeptide was bound by MAbs 3AD6, 3DB2, and 5BB1-2 and released by the SDS treatment (Fig. 5, lanes 1 to 3). Both of the MAbs which inhibited the reaction between *B. loescheii* and *A. israelii*, 2AA1 and 4AC3, released a 45-kDa polypeptide when the immune complex was treated with SDS and 2-mercaptoethanol (Fig. 5, lanes 4 and 5). The presence of the faint 45-kDa bands in lanes 1, 2, and 3, 75-kDa bands in lanes 4 and 5, and 15-kDa bands in all five lanes of Fig. 5 may be due to nonspecific binding, since CHAPS at a concentration of 0.3% or higher in the reaction mixture markedly diminished these faint bands.

A high-speed supernatant of ultrasonically disrupted cells of *B. loescheii* and the material eluted from a MAb 5BB1-2 affinity column which contained a single protein band on SDS-PAGE by Coomassie blue staining (data not shown) were applied to nondenaturing anionic polyacrylamide gels. After electrophoresis, the proteins were visualized by treating immunoblots with the 5BB1-2 or 3AD6 and an anti-mouse IgG staining system. Under native conditions, both MAbs recognized a single protein band in the high-speed supernatant and the material eluted from the 5BB1-2 affinity column (Fig. 6); the immunoreactive bands all migrated with an R_f of 0.13, suggesting that the two MAbs are specific for the same protein.

DISCUSSION

The observations (i) that rabbit anti-*B. loescheii* PK1295 serum blocked its coaggregation with both of its partners, *S. sanguis* 34 and *A. israelii* PK14 (15) and (ii) that rabbit anti-*Capnocytophaga gingivalis* serum inhibited its coaggregation with *A. israelii* PK16 (6) indicated that it would be possible to raise MAbs that specifically block adherence functions. The criterion used to identify and select anti-adhesin MAbs, namely, the inhibition of coaggregation, differentiates this study from previous attempts to identify the fimbria-associated adhesins found on gram-negative bacteria, i.e., the entero- and uropathogenic strains of *Escherichia coli* (4, 12). In the latter studies, the salient criterion used to evaluate and select hybridoma cell lines for further study was based on either ELISA scores or agglutination of cells at high antibody dilutions.

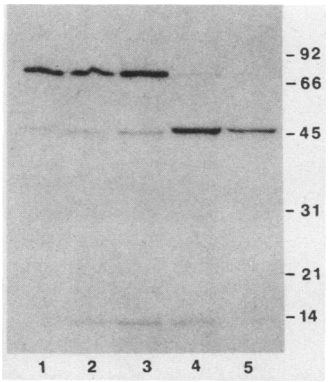

FIG. 5. Autoradiograph of ^{125}I-labeled proteins immunoprecipitated from culture supernatants by five MAbs. Lanes 1 to 3 contain proteins bound by the *S. sanguis* adhesin-specific MAbs (3AD6, 3DB2, and 5BB1-2, respectively). Lanes 4 and 5 contain proteins precipitated by the *A. israelii* adhesin-specific MAbs (2AA1 and 4AC3, respectively). The numbers on the right indicate the migration positions of molecular weight standards (10^3).

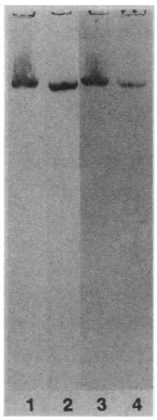

FIG. 6. Demonstration of mutual antigenic specificity of MAbs 5BB1-2 and 3AD6 for the *S. sanguis*-specific adhesin by immunoblot analysis of native gels. Lanes 1 and 3 contain ultrasonically disrupted fimbrial preparation (50 μg of protein added); lanes 2 and 4 contain purified adhesin eluted from a MAb 5BB1-2 affinity column (1 μg of protein per lane). Lanes 1 and 2 were developed with MAb 3AD6; lanes 3 and 4 were developed with MAb 5BB1-2.

Our screening strategy yielded four MAbs that inhibited the *B. loescheii-S. sanguis* interaction. Two MAbs, 5BB1-2 and 3BD2, failed to recognize any of the fimbria-associated polypeptides transferred onto immunoblots after denaturing SDS-PAGE. The other two MAbs, 3AD6 and 5DB5, readily reacted with a 75-kDa polypeptide band on the same immunoblots. It was assumed that the former group of inhibitory MAbs did not react with the 75-kDa polypeptide on immunoblots because they recognized a secondary or tertiary structure of the *S. sanguis*-specific adhesin which was obliterated or modified by denaturing PAGE. The failure of MAbs 2AA1 and 4AC3 to recognize any polypeptide on immunoblots was rationalized in a similar fashion. However, it was possible to establish the antigenic specificity of each MAb. By using samples of a radiolabeled culture supernatant that contained both adhesin activities and fimbriae, each of the MAbs was allowed to react with its antigen. When the antigen was freed from the immune complex, it was found that all of the antibodies that inhibited the interaction between *B. loescheii* and *S. sanguis* had specifically reacted with a 75-kDa protein, whereas the clones that blocked the *B. loescheii-A. israelii* interaction had bound to a 45-kDa polypeptide. The data show that our original speculations regarding the nature and size of the two adhesin monomers were relatively accurate (15). Although we have attributed the minor bands seen in Fig. 5 (lanes 1 to 5) to nonspecific adsorption, we cannot rule out the possibility that the two adhesins are associated with one another on the cell surface. In a previous study, we described several coaggregation-defective mutants which possessed fimbriae that were indistinguishable by electron microscopy from the parent strain (15). The data presented here demonstrate that the adhesins are fimbria associated; however, it is not yet clear whether the adhesins are integral parts of the fimbrial subunit or distinct entities.

Curiously, the agglutinating MAbs uniformly failed to inhibit coaggregation with either partner, whereas the coaggregation-inhibiting MAbs were incapable of agglutinating

intact *B. loescheii* cells. The sharp distinction between the properties of the agglutinating and coaggregation-inhibiting MAbs has a parallel observed in two previous studies (6, 15). In both instances, adsorption of polyclonal antisera with Cog mutants produced sera with excellent coaggregation-inhibiting properties that had lost the ability to agglutinate the cells used as immunogens. Results from the ELISAs address the distinction between the agglutinating and blocking MAbs more directly. Although the concentrations of both coaggregation-blocking and agglutinating MAbs required to produce half-maximal effects were of the same order of magnitude in ELISAs (with the exceptions of 5BB1-2 and 4AC3), marked differences were observed in the rate and final intensity of color development. Agglutinating MAbs caused higher concentrations of chromophore to be released more rapidly than did adhesin-specific MAbs. Since a constant amount of antigen (saturating levels) was added to all microtiter wells, the difference in the immunological responses between the two groups of MAbs may be a manifestation of differences in the total number of antigenic binding sites available for the antibody interaction.

The comparatively low concentrations of MAb required to completely inhibit coaggregations between *B. loescheii* and its two partners, *S. sanguis* (Fig. 3) and *A. israelii* (Fig. 4), argues strongly that the MAbs recognize epitopes at or near the binding sites of the two adhesins. The fact that Fab fragments are effective at the same or lower concentrations serves to buttress this argument. The sixfold increase in efficacy of the 5BB1-2 Fab preparation is probably due to (i) the higher state of purity of the Fab as compared with the MAb and (ii) the greater molar concentration of the Fab per microgram of protein.

Experiments are already under way, using the MAbs described here, to determine the number and location of the adhesins on the surface of *B. loescheii*. We are most interested to learn whether the two antigenically distinct adhesins are, in fact, present in large or small numbers and how they are associated with the fimbriae.

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