

OPSONIZATION OF BACTEROIDES BY THE
ALTERNATIVE COMPLEMENT PATHWAY
RECONSTRUCTED FROM ISOLATED PLASMA PROTEINS

BY ANN B. BJORNSON,* PATRICIA I. MAGNAFICHI,*
ROBERT D. SCHREIBER,‡ AND H. STEPHEN BJORNSON§

*From the *Division of Immunology, James N. Gamble Institute of Medical Research, Cincinnati, Ohio 45219; the †Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037; and the §Division of Surgical Infectious Diseases, Department of Surgery, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267*

Since the discovery of properdin by Pillemer et al. (1) in 1954, it has been recognized that the alternative pathway of the complement system plays a major role in natural resistance against microbial infection. Early studies demonstrated that the alternative pathway functioned in bacteriolysis (2), neutralization of viruses (3), and protozoal killing, (4, 5). The critical role of the alternative pathway in bacterial opsonization was documented later in classic studies by Wood and his co-workers (6–8) utilizing pneumococci. These studies suggested that natural antibodies and the alternative pathway interacted functionally to facilitate opsonization of pneumococci (8). Since then, several bacterial species have been shown to activate the alternative pathway in serum and to utilize this pathway during opsonization by serum (reviewed in reference 9). However, little is known about the molecular dynamics of alternative pathway activation by bacteria, the role of antibodies and other serum factors in this process, and the relative contributions of the alternative pathway and auxiliary factors to opsonic recognition by phagocytic cells.

We have used a model system comprising the six isolated proteins of the alternative pathway to study the opsonic capacity of this pathway for *Bacteroides fragilis* and *Bacteroides thetaiotaomicron* in the absence of other factors. *B. fragilis* and *B. thetaiotaomicron* are normal components of the intestinal microflora and have a symbiotic relationship with the host under normal circumstances. Clinical infections due to these bacteria usually occur as a result of perturbation of the intestinal mucosa with liberation of bacteria into the surrounding tissues. Previous studies from our laboratory have demonstrated that clinical isolates of *B. fragilis* and *B. thetaiotaomicron* are resistant to the bactericidal activity of serum but are readily opsonized by serum, which facilitates phagocytosis and intracellular killing by polymorphonuclear leukocytes in vitro (10, 11). Involvement of the alternative pathway in opsonization of these bacteria has been demonstrated

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using sera depleted of alternative pathway proteins and the respective isolated proteins to restore opsonic activity to these sera (10). The present study demonstrates that activation of the alternative pathway by *B. fragilis* and *B. thetaiotaomicron* and resultant C3 deposition on the bacterial surfaces does not require auxiliary serum factors, but the effector phase of opsonization of these bacteria involving adherence, uptake, and killing of the bacteria by polymorphonuclear leukocytes is dependent on such factors.

Materials and Methods

Bacterial Strains and Growth Conditions. *B. fragilis* 1365 and *B. thetaiotaomicron* 1343 were clinical isolates used in our previous studies (10). Stock cultures were maintained in thioglycolate medium (Difco Laboratories, Detroit, MI) in frozen form at -70°C . Cultures were thawed and inoculated into broth consisting of equal parts of trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD) and brain-heart infusion broth (Difco Laboratories) supplemented with 0.05% thioglycolate (Difco Laboratories). For preparation of radiolabeled bacteria, broth was supplemented with $10\ \mu\text{Ci/ml}$ of L-[^3H]amino acid mixture (New England Nuclear, Boston, MA). Cultures were incubated for 20 h at 37°C in an anaerobic glove box (Coy Manufacturing Products, Ann Arbor, MI), which was monitored to contain 85% nitrogen, 10% hydrogen, 5% carbon dioxide, and not more than 20 parts per million of oxygen. Bacteria were washed and resuspended in HBSS (M. A. Bioproducts, Walkersville, MD) containing 0.1% gelatin (Difco Laboratories) (HBG)¹; this solution was boiled for 10 min before use to remove oxygen.

Isolation of Complement Proteins and IgM. C3 was prepared from human plasma by the method of Tack et al. (12). Trace contaminants were removed by chromatography on anti-IgG Sepharose 4B, anti-IgA Sepharose 4B, and anti-C5 Sepharose 4B equilibrated with isotonic veronal-buffered saline (VBS), pH 7.4. Antisera to IgG (γ -chain-specific), IgA (γ -chain-specific), and C5 were obtained from Behring Diagnostics, La Jolla, CA, and IgG fractions of these antisera were prepared by ammonium sulfate precipitation (13). The IgG fractions were coupled to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) by cyanogen bromide activation (14).

Factor B was prepared by a minor modification of the method of Gotze and Muller-Eberhard (15). Human serum was precipitated with 40% ammonium sulfate (wt/vol) at 4°C , and the precipitate was discarded. The ammonium sulfate concentration in the supernatant was raised to 68%. The resulting precipitate was solubilized and then chromatographed according to the original procedure, except DEAE Sephacel (Pharmacia Fine Chemicals) was substituted for DEAE-cellulose. As a final step in the purification procedure, active fractions from the DEAE Sephacel column were chromatographed on anti-IgG Sepharose 4B.

Factor D was prepared by the method of Davis et al. (16), except CM-Sepharose CL-6B (Pharmacia Fine Chemicals) was substituted for CM-Sephadex. Final purification was achieved by chromatography on a $2.6 \times 90\text{-cm}$ column of Sephadex G-75 (Pharmacia Fine Chemicals).

Factor H was harvested from the DEAE Sephacel column used in purification of C3. Fractions containing factor H were dialyzed at 4°C against 0.008 M EDTA, pH 5.5, to precipitate euglobulin. The precipitate was dissolved in VBS containing 0.01 M EDTA and 0.1% sodium azide and then chromatographed on a $5.0 \times 44\text{-cm}$ column of Bio-Gel A-1.5 M (Bio-Rad Laboratories, Inc., Richmond, CA) equilibrated with the same buffer. Factor H containing fractions were further purified by chromatography on anti-IgG Sepharose 4B.

Factor I was prepared by successive chromatography of human plasma on anti-factor I Sepharose 4B, DEAE Sephacel, and anti-IgG Sepharose 4B (17). Trace transferrin was removed by chromatography on anti-transferrin Sepharose 4B. Antiserum to factor I was raised in a goat by repeated subcutaneous injections of purified factor I incorporated into

¹ Abbreviations used in this paper: HBG, HBSS-gelatin buffer; VBS, veronal-buffered saline.

CFA (Difco Laboratories). Antiserum to human transferrin was obtained from Behring Diagnostics. IgG fractions of these antisera were prepared by ammonium sulfate precipitation (13), and the IgG fractions were coupled to Sepharose 4B by cyanogen bromide activation (14).

Properdin was prepared by successive chromatography of 150 ml of fresh human serum on a 5.0×36 -cm column of QAE-Sephadex A-50 (Pharmacia Fine Chemicals) and a 50-ml column of anti-IgG Sepharose 4B. The conditions described by Gotze et al. (18) were used for the QAE-Sephadex chromatography, except the pH of the equilibrating buffer was adjusted to 8.5 rather than 8.0.

IgM was prepared from pooled normal human serum by polyethylene glycol precipitation, affinity chromatography on anti-human IgM Sepharose 4B, and subsequent chromatography on Sephacryl S-300 (19). To maximize removal of IgG, active fractions from the Sephacryl column were chromatographed on anti-human IgG Sepharose 4B. The IgG fraction of anti-human IgM (γ chain-specific; Behring Diagnostics) was prepared and coupled to Sepharose 4B as described for other immunoabsorbents.

Active fractions of C3, factor B, factor H, factor I, and IgM were identified by double immunodiffusion using monospecific antisera. Factor D and properdin containing fractions were identified by a hemolytic assay using human serum depleted of factor D or properdin, respectively. Factor D depleted serum was prepared as described by Lesavre et al. (20). Properdin-depleted serum was prepared by chromatography of 15 ml of serum containing 0.01 M EDTA on a 30 ml column of anti-properdin Sepharose 4B. The antiserum to human properdin used for preparation of the immunoabsorbent was obtained from Atlantic Antibodies, Scarborough, ME; coupling of the IgG fraction to Sepharose 4B was performed by cyanogen bromide activation (14).

Isolated proteins were concentrated by ultrafiltration using YM-10 (factor D) or YM-30 membranes (other proteins) and stirred ultrafiltration cells from Amicon Corp. (Danvers, MA). Proteins were dialyzed overnight against VBS and stored in small aliquots at 4°C (properdin) or -70°C (all other proteins). IgM was centrifuged at 10,000 *g* for 5 min at 4°C to remove possible aggregates before use in the experiments.

Measurement of Protein. Protein in isolated preparations was quantitated by the method of Lowry et al. (21) using crystalline BSA as the reference standard.

Purity of Isolated Proteins. Purity of isolated protein preparations was assessed by SDS-PAGE, double immunodiffusion, and ELISA. SDS-PAGE was performed by the method of Laemmli (22) in a 10% polyacrylamide slab gel. Three to 5 μ g of protein were applied to the gel. Molecular weight markers run simultaneously were phosphorylase a, BSA, aldolase, and α -chymotrypsinogen A. All samples except properdin were heated for 45 min at 37°C before application; properdin was boiled for 3 min. Samples were reduced by inclusion of 0.1 M dithiothreitol. The gel was fixed overnight in an aqueous solution of 25% propanol and 10% glacial acetic acid, stained with 0.25% Coomassie Brilliant Blue R-250, and destained with an aqueous solution of 7.5% glacial acetic acid and 5% methanol.

Double immunodiffusion was carried out at room temperature for 18 h in 0.6% agarose in 0.01 M PBS, pH 7.0, using antisera to human Clq, C4, C2, C3, C5, factor B, factor I, factor H, properdin, IgG, IgM, IgA, albumin, fibronectin, transferrin, and whole human serum. Final isolated preparations were tested at physiological concentration. Antisera to human C3, C4, factor H, and factor I were prepared in goats by repeated subcutaneous injections of isolated proteins incorporated into Freund's complete adjuvant; isolated C3, factor H, and factor I were prepared as described above, and C4 was obtained from Cordis Laboratories, Inc., Miami, FL. Other antisera were obtained commercially from Behring Diagnostics (anti-IgG, IgA, IgM, Clq, C5, factor B, albumin, fibronectin, transferrin, and whole human serum), Atlantic Antibodies (anti-properdin), and California Immunodiagnosics Inc., San Marcos, CA (anti-C2). Antisera to the immunoglobulins were γ chain-specific.

An ELISA was used as a highly sensitive indicator of IgG contamination of isolated complement preparations (23). An IgG standard was prepared by precipitation of human serum with ammonium sulfate and chromatography on DEAE Sephacel (24); this prepa-

ration was highly purified as assessed by SDS-PAGE and double immunodiffusion as described above. The inner wells of microtiter plates were coated for 18 h at 4°C with 100 µl of IgG standard (10–0.001 µg/ml), IgM (10 µg/ml), or a mixture of the six isolated complement proteins at physiological concentrations in 0.1 M sodium bicarbonate/carbonate buffer, pH 9.6. The plates were washed extensively with 0.15 M phosphate buffer, pH 7.4, containing 0.5 M sodium chloride and 0.05% Tween 20 (PBS-tween). 100 µl of alkaline phosphatase-labeled affinity-purified anti-human IgG (Sigma Chemical Co., St. Louis, MO) diluted 1:1,000 in PBS-tween was added, and the plates were incubated for 2 h at room temperature. After additional washing with PBS-tween, 100 µl of *p*-nitrophenyl phosphate (Sigma Chemical Co.) at a concentration of 1 mg/ml in 0.05 M sodium bicarbonate/carbonate buffer, pH 9.8, containing 0.001 M magnesium chloride was added to the wells. The plates were incubated at room temperature for 15 min, and the reaction was stopped by addition of 50 µl of 3 N sodium hydroxide. The absorbance at 405 nm was measured using an ELISA reader (Litton Bionetics Inc., Charleston, SC).

Radioiodination of Proteins. C3 and IgM were radioiodinated by the lactoperoxidase method using Enzymobeads from Bio-Rad Laboratories and the manufacturer's general directions. For radioiodination of C3, purified C3 (100 µg), 300 µCi of sodium ¹²⁵I (Amersham Corp., Arlington Heights, IL), 25 µl of Enzymobead reagent, 20 µl of 1% β-D-glucose, and 40 µl of 0.2 M sodium phosphate buffer, pH 7.2, in a total volume of 100 µl were incubated for 30 min on ice. For radioiodination of IgM, 500 µg of purified IgM and 250 µCi of sodium ¹²⁵I were used. Free iodine was removed by chromatography on Sephadex G-25 (Pharmacia Fine Chemicals) equilibrated with VBS. Specific activity of the radioiodinated C3 preparations ranged from 0.47 to 1.73 µCi/µg. Specific activity of radioiodinated IgM was 0.13 µCi/µg. Titration of C3 hemolytic activity (25) showed that hemolytic activity in radioiodinated C3 was equivalent to that in nonlabeled C3 on a weight basis; C3 hemolytic activity was titrated using complement components and erythrocyte intermediate from Cordis Laboratories, Miami, FL. Radioiodinated and nonlabeled IgM also had similar functional activity as assessed by comparing the ability of these preparations to opsonize *B. thetaiotaomicron* together with the six isolated alternative pathway proteins.

Preparation of Isolated Complement Mixtures. Isolated complement proteins were mixed at physiological concentrations as described previously (26). C3, factor H, and factor I were incubated for 30 min at 37°C. Factor B, factor D, and properdin or combinations of these proteins were then added. Final protein concentrations were: 1,200 µg/ml C3; 200 µg/ml factor B; 2 µg/ml factor D; 500 µg/ml factor H; 34 µg/ml factor I; and 20 µg/ml properdin. The mixtures were supplemented with 0.005 M magnesium chloride and 0.0015 M calcium chloride before use in the experiments.

Serum Lacking Classical Pathway Activity (EGTA-serum). Pooled human serum was treated with 0.01 M EGTA and 0.01 M magnesium chloride to block classical pathway activity and allow selective complement activation via the alternative pathway. Total hemolytic complement was not detected in this serum, confirming that classical pathway activity was effectively inhibited. Total hemolytic complement was measured by the method of Mayer (27).

Functional Testing of Isolated Proteins. C3 deposition on rabbit and sheep erythrocytes in mixtures of the isolated complement proteins and in serum was performed by the method of Schreiber et al. (28). Isolated protein mixtures or serum were supplemented with radioiodinated C3 and centrifuged at 10,000 *g* for 3 min at 4°C to remove possible aggregates. The amount of labeled C3 added to the serum was adjusted so that the final samples analyzed contained ~10⁵ total cpm. Equal parts of protein mixture or serum and erythrocytes (10⁹ cells/ml) were incubated at 37°C. At various time intervals, aliquots were removed and rapidly centrifuged through 20% sucrose in VBS to separate cell-bound and free radioactivity. The number of C3 molecules bound per erythrocyte was calculated by multiplying the percent of C3 deposition by the number of C3 molecules added per erythrocyte.

To detect possible fluid phase consumption of C3, mixtures of the six isolated complement proteins were incubated for 15 min at 37°C or 4°C, and C3 hemolytic activity was

titrated in these mixtures. Results were compared with those obtained with a control C3 preparation that was not incubated.

Quantitation of C3 Deposition on Bacteria. Isolated protein mixtures or serum were supplemented with radioiodinated C3 and centrifuged as described above. Equal parts of protein mixture or serum and bacteria (10^8 CFU/ml) were incubated at 37°C . At various times intervals, aliquots were removed and rapidly centrifuged through 20% sucrose in VBS to separate bacterial-bound and free radioactivity. Results were expressed as C3 molecules bound per CFU, which was calculated by multiplying the percent C3 deposition by the number of C3 molecules added per CFU.

Opsonization of the Bacteria. Equal parts of ^3H -labeled bacteria (10^8 CFU/ml) and isolated complement mixture or serum were incubated at 37°C in the anaerobic glove box. IgM (750 $\mu\text{g}/\text{ml}$) was included in the reaction mixtures in some experiments. After 15, 30, or 45 min of incubation, the bacteria were washed once and resuspended in deoxygenated HBG except where otherwise indicated in the Results.

Preparation of Polymorphonuclear Leukocytes. Polymorphonuclear leukocytes from healthy adult donors were isolated by centrifugation of heparinized blood on Hypaque-Ficoll (29). Contaminating erythrocytes were removed by hypotonic lysis with 0.2% sodium chloride; isotonicity was restored by addition of an equal volume of 1.6% sodium chloride. Leukocytes were washed and resuspended in deoxygenated HBG. Suspensions contained 98% or more polymorphonuclear leukocytes.

Measurements of Bacterial Uptake and Killing. Opsonized bacteria and polymorphonuclear leukocytes at final concentrations of 2.5×10^7 cells or CFU/ml were rotated at 37°C in the anaerobic glove box. After 5, 10, and 20 min of incubation, bacterial uptake and killing were measured in duplicate using minor modifications of previously published methods (11). For measurement of bacterial uptake, aliquots were centrifuged at 190 g for 5 min at 4°C . The leukocytes were washed three times with cold deoxygenated HBG. Leukocyte pellets and samples removed before incubation for measurement of input counts per minute were solubilized and counted. For measurement of killing, aliquots removed before and after incubation were serially diluted in deoxygenated distilled water and plated by the pour plate method in agar consisting of equal parts of trypticase soy agar (BBL Microbiology Systems) and brain-heart infusion agar (Difco Laboratories) supplemented with 0.5% yeast extract (BBL Microbiology Systems) and 0.05% thioglycolate. Percent uptake, reflecting both adherent and ingested bacteria, was calculated by dividing the counts per minute in the leukocyte pellets by the input counts per minute and multiplying by 100. Percent killing was calculated by the formula $(a - b)/a \times 100$, where a and b were equal to surviving CFU/ml before and after incubation, respectively.

In some experiments, direct killing of the bacteria by pooled human serum was determined. Bacteria (10^8 CFU/ml) were incubated at 37°C with 50% serum (vol/vol), and surviving bacteria were enumerated at 30 and 60 min as described above.

Measurement of Bacterial Adherence to Polymorphonuclear Leukocytes. Two methods were used to measure adherence of opsonized bacteria to polymorphonuclear leukocytes. In the first method, polymorphonuclear leukocytes were treated for 15 min at room temperature with 10 $\mu\text{g}/\text{ml}$ of cytochalasin B to inhibit ingestion. Cytochalasin B-treated polymorphonuclear leukocytes and opsonized bacteria were rotated at 37°C at final concentrations of 2.5×10^7 cells or CFU/ml. After 5, 10, and 20 min of incubation, bacterial uptake was measured as described in the preceding section, except the leukocytes were washed twice rather than three times before solubilization and counting.

In the second method, polymorphonuclear leukocytes were incubated for 30 min at 4°C with 10 $\mu\text{g}/\text{ml}$ cytochalasin B. Equal volumes of opsonized bacteria (2.5×10^7 CFU/ml) and polymorphonuclear leukocytes ($2.5 \times 10^6/\text{ml}$) were incubated with gentle tumbling at 4°C . After 30 min and 18 h of incubation, 30 μl aliquots were removed, and 5 μl of 0.015% acridine orange in HBSS was added to each aliquot. Sealed wet mounts were prepared, and the number of bacteria adhering to 100 polymorphonuclear leukocytes was counted using a Zeiss fluorescent microscope (Carl Zeiss, Inc., Thornwood, NY). Preliminary experiments confirmed that cytochalasin B inhibited ingestion of serum opsonized bacteria by polymorphonuclear leukocytes under both sets of conditions.

Analysis of Bacteria-bound C3 Fragments. Isolated protein mixtures or serum were supplemented with 2.8×10^7 cpm/ml radioiodinated C3 and centrifuged at 10,000 *g* for 3 min at 4°C to remove possible aggregates. Equal parts of bacteria (10^8 CFU/ml) and protein mixture or serum in a volume of 500 μ l were incubated for 30 min at 37°C. The bacteria were then washed twice with deoxygenated HBSS. Bacteria-bound C3 was extracted, quantitated, and analyzed as described by Weis et al. (30). Briefly, the bacterial pellets were incubated for 60 min at 37°C in 0.1 M carbonate/bicarbonate buffer, pH 11, containing 0.025 M methylamine and 1% SDS. The bacteria were centrifuged, and radioactivity in the pellets and supernatants was counted. The percent extracted C3 was calculated by dividing the radioactivity in the supernatants by the sum of the radioactivity in the pellets and supernatants and then multiplying by 100. Supernatants containing extracted C3 were subjected to 6–12% linear gradient SDS-PAGE under reducing conditions, and C3 cleavage fragments were analyzed by autoradiography. Prestained protein standards from Bethesda Research Laboratories, Gaithersburg, MD, were used in these analyses.

Binding of IgM to the Bacteria. Nonlabeled IgM was supplemented with radioiodinated IgM so that the final samples analyzed contained $\sim 10^5$ total cpm. Bacteria (10^8 CFU/ml) were incubated for 30 min at 37°C with 750 μ g/ml of the trace-labeled IgM. After incubation, triplicate samples were rapidly centrifuged through 20% sucrose in VBS, and bacterial-bound and free radioactivity was measured. Results were expressed as IgM molecules bound per CFU and were calculated by multiplying the percent of bound IgM by the number of IgM molecules added per CFU.

Results

Purity and Functional Activity of Isolated Complement Proteins. Isolated preparations of the six proteins of the alternative pathway used in our investigation were highly purified as assessed by SDS-PAGE (Fig. 1). The isolated proteins reacted in double immunodiffusion only with homologous antisera and formed

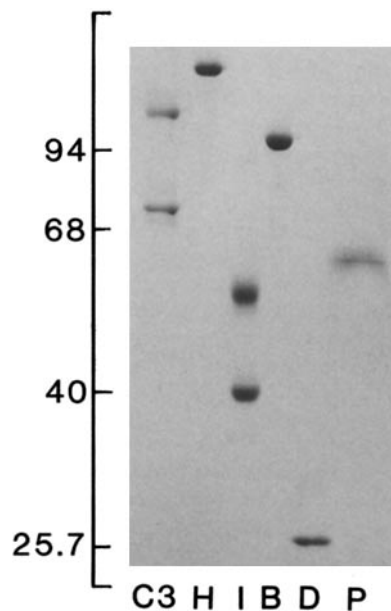


FIGURE 1. SDS-PAGE of isolated C3 (C3), factor H (H), factor I (I), factor B (B), factor D (D), and properdin (P) under reducing conditions. Numbers on the left refer to the positions of standard proteins having the indicated $M_r \times 10^{-3}$.

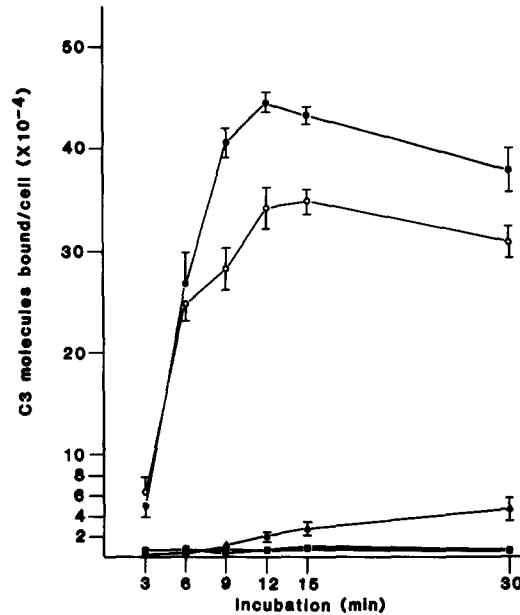


FIGURE 2. Kinetics of ^{125}I -C3 deposition on rabbit erythrocytes during incubation with the alternative complement pathway reconstructed from isolated proteins. Rabbit erythrocytes were incubated at 37°C with a complete mixture of the six isolated complement proteins (*solid circles*), a mixture of five proteins lacking factor D (*solid squares*), or properdin (*solid triangles*), the complete mixture heat-inactivated at 56°C for 30 min (*open squares*), or EGTA-serum (*open circles*). At the specified time intervals, the number of C3 molecules bound per erythrocyte was quantitated. Data are expressed as mean \pm SEM of three to four determinations.

a single precipitin line with antiserum to whole human serum, except factor D which was nonreactive.

The alternative pathway was reconstructed by mixing the six isolated complement proteins at physiological concentrations. This mixture was devoid of IgG as assessed by the ELISA; the lower limit of detection of IgG in this assay was $0.01 \mu\text{g/ml}$ of IgG. Functional activity of the isolated proteins was determined by comparing ^{125}I -C3 deposition on rabbit erythrocytes during 30 min of incubation at 37°C with the complement mixture and EGTA-serum. C3 deposition was also measured during incubation of the erythrocytes with mixtures of five isolated proteins (lacking factor D, factor B, or properdin) and with a mixture of the six proteins heat-inactivated at 56°C for 30 min. The kinetics of C3 deposition on rabbit erythrocytes were similar during incubation with the complete protein mixture and EGTA-serum (Fig. 2). The magnitude of the response was 1.3-fold greater in the isolated mixture, a finding consistent with previous data comparing C3 deposition on particulate activators of the alternative pathway in isolated complement mixtures and serum under conditions similar with those used in our study (28, 31). Deletion of properdin from the protein mixture markedly reduced C3 deposition, and deletion of factor D resulted in a background level of C3 deposition equal to that observed with the heat-inactivated protein mixture. Similar results were obtained with the mixture of complement proteins lacking factor B (data not shown). These data confirm previous obser-

TABLE I
*Lack of Spontaneous C3 Consumption in the Alternative Complement
 Pathway Reconstructed from Isolated Proteins*

Protein mixture	Temperature of incubation	C3 hemolytic activity (CH ₅₀)
	°C	U/ml
Complete	37	3,040
Complete	4	2,580
C3	Unincubated	2,520

Complete mixtures of the six isolated complement proteins were incubated for 15 min at the specified temperatures, and C3 hemolytic activity was titrated in these mixtures and in an equivalent amount of C3 alone. Data are from a representative experiment.

vations demonstrating an absolute requirement for factors B and D for alternative pathway activation and augmentation of this process by properdin (26).

Two approaches were used to rule out spontaneous consumption of C3 in the alternative pathway reconstructed from isolated proteins. First, complete mixtures of the six isolated complement proteins were incubated for 15 min at 37°C or 4°C, and C3 hemolytic activity in these mixtures was compared with that in an equivalent amount of C3 alone. Hemolytic activity was equivalent in the protein mixtures and the control C3 preparation, ruling out C3 consumption in the fluid phase (Table I). Secondly, ¹²⁵I-C3 deposition was measured on sheep erythrocytes, a nonactivator of the alternative pathway, during 30 min of incubation at 37°C with the complete protein mixture (untreated and heat-inactivated), EGTA-serum, and untreated serum. 10^4 C3 molecules were deposited per erythrocyte after 30 min of incubation with both protein mixtures and EGTA-serum. In contrast, 2.0×10^5 C3 molecules were deposited per erythrocyte after incubation with untreated serum (containing both classical and alternative complement pathway activity). These data further negate fluid-phase C3 consumption in the complete mixture of isolated complement proteins.

Alternative Pathway Activation by Bacteroides. Serum opsonization of the isolates of *B. fragilis* and *B. thetaiotaomicron* used in our investigation has been previously shown to involve the alternative pathway (10). To determine the capacity of these bacteria to activate the alternative pathway, ¹²⁵I-C3 deposition on the bacteria was measured during 60 min of incubation at 37°C with the complete mixture of six isolated complement proteins or EGTA-serum. Bacteria incubated with a mixture of five complement proteins lacking factor D and with a heat-inactivated complete protein mixture served as the negative controls. C3 deposition on the *Bacteroides* strains was demonstrated during incubation with the complete protein mixture with maximal levels of $1.4-1.7 \times 10^5$ C3 molecules per CFU occurring by 45 min of incubation (Fig. 3). Maximal deposition of $1.9-2.7 \times 10^5$ C3 molecules per CFU occurred by 30 min of incubation with EGTA-serum. C3 deposition was minimal in the incomplete protein mixture (lacking factor D) and the heat-inactivated protein mixture. These data demonstrate that *B. fragilis* and *B. thetaiotaomicron* are potent activators of the alternative pathway

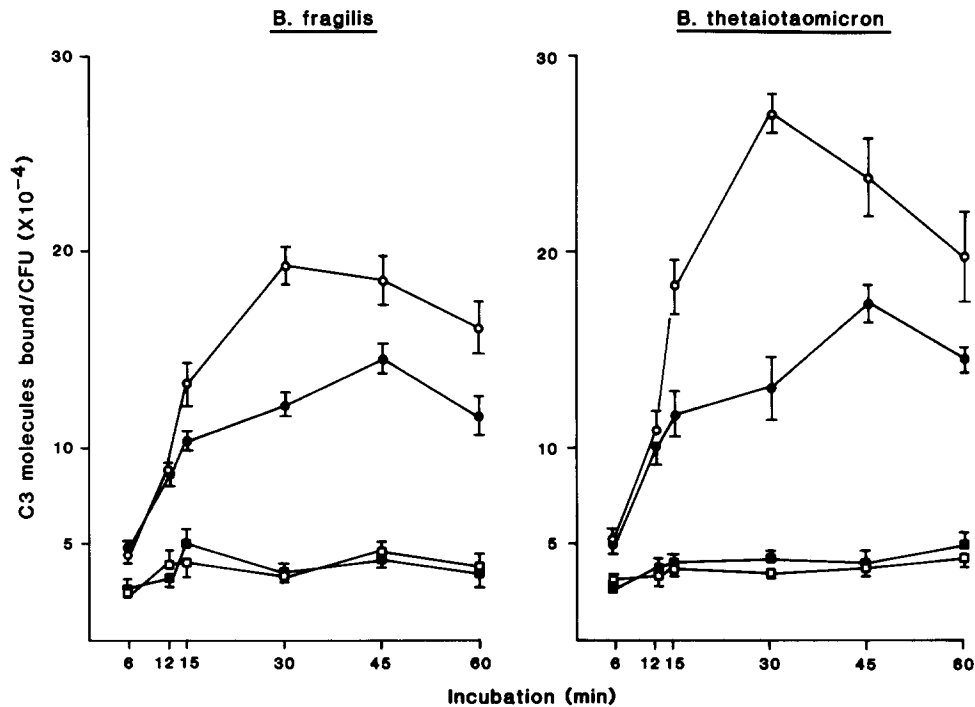


FIGURE 3. ^{125}I -C3 deposition on *B. fragilis* and *B. thetaiotaomicron* during incubation with isolated protein mixtures or EGTA-serum. Bacteria were incubated at 37°C with a complete mixture of the six isolated complement proteins (untreated [solid circles] or heat-inactivated at 56°C for 30 min [open squares]), a mixture of five proteins lacking factor D (solid squares), or EGTA-serum (open circles). At the specified time intervals, the number of C3 molecules bound per CFU was quantitated. Data are expressed as mean \pm SEM of five to nine determinations.

in the absence of other factors. The data suggest that auxiliary factors in serum augment activation of the alternative pathway by the *Bacteroides* strains.

Opsonic Capacity of the Alternative Pathway for Bacteroides. To determine the opsonic capacity of the alternative pathway for the *Bacteroides* strains, the bacteria were opsonized with isolated protein mixtures or EGTA-serum, washed, and incubated at 37°C with polymorphonuclear leukocytes. Bacterial uptake and killing were measured during 20 min of incubation. The conditions used for opsonizing the bacteria were identical to those used for measurements of C3 deposition, so that the results could be compared. Bacteria were opsonized with the various complement sources for 30 min at 37°C , because C3 deposition on the bacteria was maximal or near maximal at this time (refer to Fig. 3). Preliminary experiments demonstrated that heat inactivation of EGTA-serum at 56°C for 30 min ablated bacterial uptake and killing, indicating that complement was essential for opsonization (data not shown). Opsonization of the bacteria with isolated protein mixtures (complete or lacking factor D) promoted minimal uptake and killing by the leukocytes, whereas 80–90% uptake and killing were observed when the bacteria were opsonized with EGTA-serum (Figs. 4 and 5). The bacteria were not susceptible to direct killing by serum (data not shown), and bacterial uptake and killing by the leukocytes were not demonstrated in the

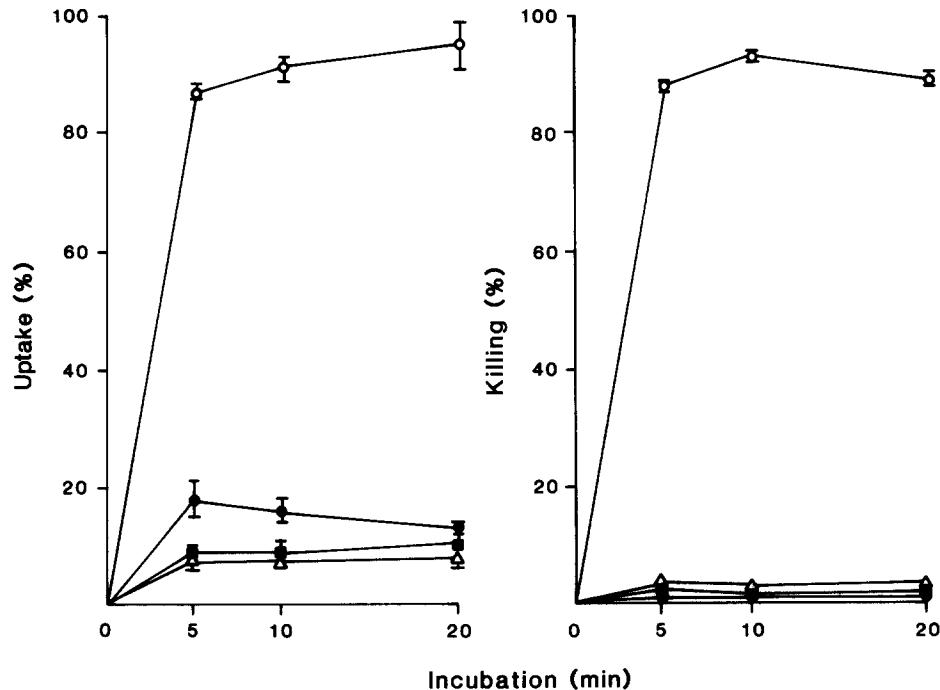


FIGURE 4. Uptake and killing by polymorphonuclear leukocytes of *B. fragilis* opsonized with isolated protein mixtures or EGTA-serum. Bacteria were incubated for 30 min at 37°C with a complete mixture of the six isolated complement proteins (solid circles), a mixture of five proteins lacking factor D (solid squares), EGTA-serum (open circles), or HBSS (open triangles). After washing, the bacteria were further incubated with polymorphonuclear leukocytes, and uptake and killing were measured at the specified time intervals. Data are expressed as mean \pm SEM of two determinations.

absence of opsonins (Figs. 4 and 5). Opsonization of the bacteria with the complement sources for 15 or 45 min yielded results similar to those obtained when the bacteria were opsonized for 30 min (data not shown). These data taken together with the results on C3 deposition demonstrate that alternative pathway activation and C3 deposition alone are not sufficient to facilitate uptake and killing of *B. fragilis* and *B. thetaiotaomicron* by polymorphonuclear leukocytes. The results suggest that auxiliary serum factors are necessary to promote these events.

Ability of the Alternative Pathway to Facilitate Adherence. For measurement of bacterial adherence to polymorphonuclear leukocytes, the leukocytes were pretreated with cytochalasin B under conditions that were shown to inhibit ingestion. In initial experiments, radiolabeled bacteria were opsonized with the complete mixture of isolated complement proteins or EGTA-serum for 30 min at 37°C, and uptake by cytochalasin B-treated polymorphonuclear leukocytes was measured during 20 min of further incubation at 37°C. Under these conditions, bacteria opsonized with isolated proteins adhered minimally to the polymorphonuclear leukocytes, whereas a high level of adherence was observed when the bacteria were opsonized with EGTA-serum (Fig. 6).

To determine whether these differences in adherence were related to quanti-

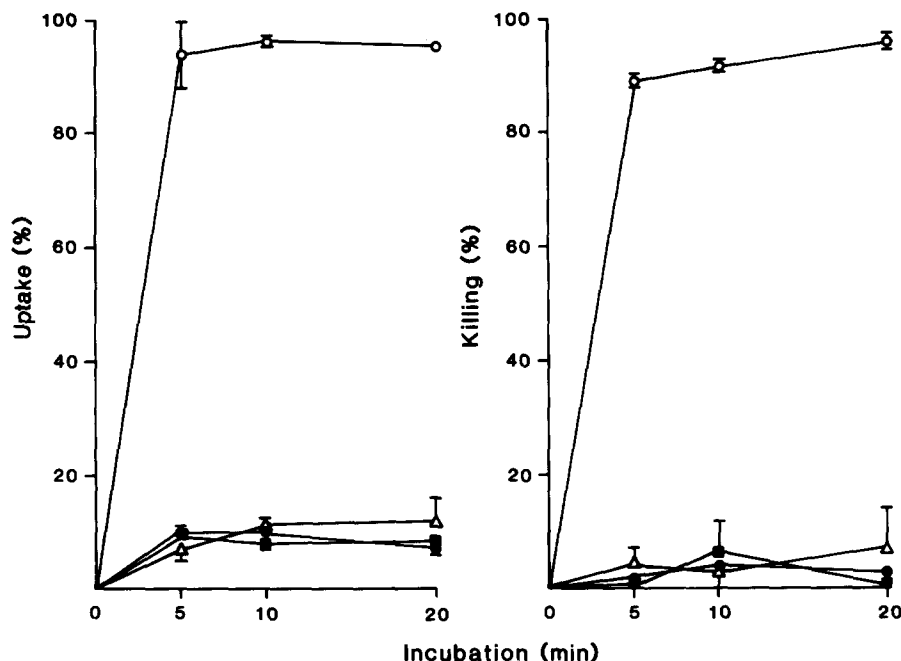


FIGURE 5. Uptake and killing by polymorphonuclear leukocytes of *B. thetaiotaomicron* opsonized with isolated protein mixtures or EGTA-serum. Experimental conditions were as outlined in Fig. 4. Data are expressed as mean \pm SEM of two determinations.

tative differences in C3 deposition on the bacteria, C3 deposition mediated by isolated proteins was increased by opsonizing the bacteria twice with these proteins. This treatment resulted in binding of 2.0×10^5 C3 molecules per CFU on *B. fragilis* and 2.7×10^5 C3 molecules per CFU on *B. thetaiotaomicron*, numbers equivalent to those bound to bacteria opsonized with EGTA-serum (refer to Fig. 3). However, only a minor increase in adherence of the bacteria to the polymorphonuclear leukocytes was observed (Fig. 6). These results indicate that the difference in opsonic capacity of isolated proteins and EGTA-serum is not solely related to the concentration of bacteria-bound C3.

Additional experiments employed more stringent conditions for measurement of adherence. In these experiments, the polymorphonuclear leukocytes were treated with cytochalasin B at 4°C, and adherence of acridine orange stained bacteria was measured by fluorescence microscopy after 30 min and 18 h of incubation at 4°C. The ratio of bacteria to polymorphonuclear leukocytes in these assays was 10:1 as compared with 1:1 in other experiments. *B. fragilis* opsonized with isolated complement proteins adhered minimally to the polymorphonuclear leukocytes under these conditions (Table II). Similarly opsonized *B. thetaiotaomicron* adhered to the polymorphonuclear leukocytes to a greater extent; however, adherence was considerably less than adherence of bacteria opsonized with EGTA-serum. These results provide additional support for the concept that the alternative pathway is inefficient in facilitating adherence of the *Bacteroides* strains to polymorphonuclear leukocytes in the absence of other factors.

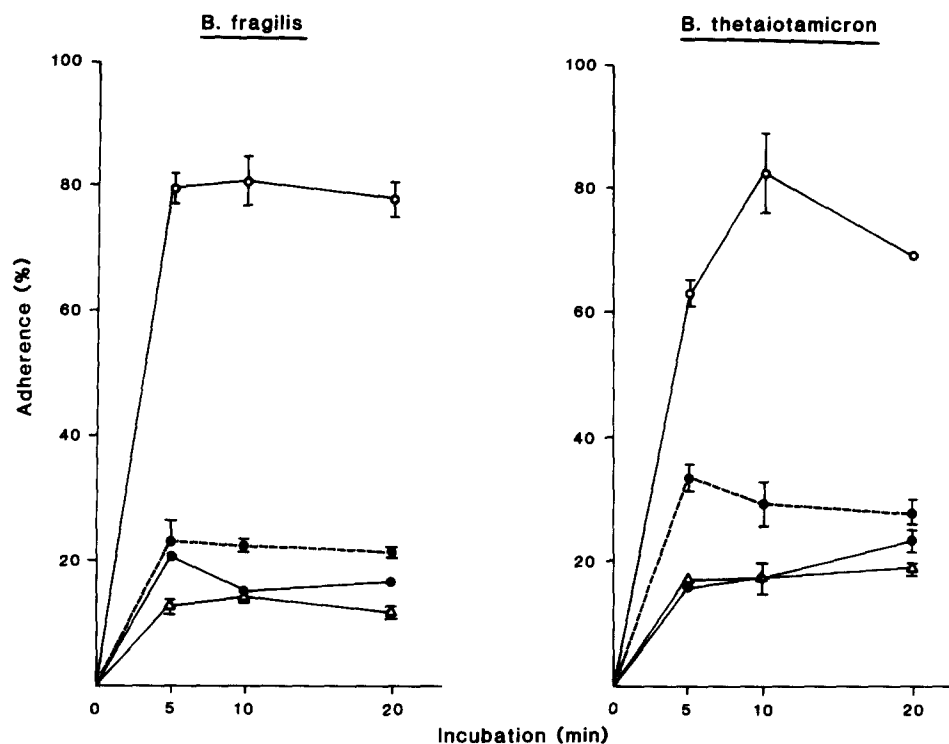


FIGURE 6. Adherence to polymorphonuclear leukocytes of *B. fragilis* and *B. thetaiotaomicron* opsonized with isolated complement proteins or EGTA-serum. Bacteria were incubated for 30 min at 37°C with a complete mixture of the six isolated complement proteins (solid circles), EGTA-serum (open circles), or HBSS (open triangles). (Dotted line) Bacteria opsonized twice with the isolated complement mixture. After washing, the bacteria were further incubated with cytochalasin B-treated polymorphonuclear leukocytes, and uptake was measured at the specified time intervals. Data are expressed as mean \pm SEM of two determinations.

TABLE II
Adherence of Opsonized Bacteroides to Cytochalasin B-treated
Polymorphonuclear Leukocytes at 4°C

Complement source used for opsonization	Adherence			
	<i>B. fragilis</i>		<i>B. thetaiotaomicron</i>	
	30 min	18 h	30 min	18 h
	%			
Isolated proteins	8	4	18	20
EGTA-serum	45	62	42	61
HBSS	4	2	2	11

Bacteria were incubated for 30 min at 37°C with a complete mixture of six isolated complement proteins, EGTA-serum, or HBSS. After washing, the bacteria were incubated with cytochalasin B-treated polymorphonuclear leukocytes at 4°C. Adherence was measured microscopically after 30 min and 18 h of incubation.

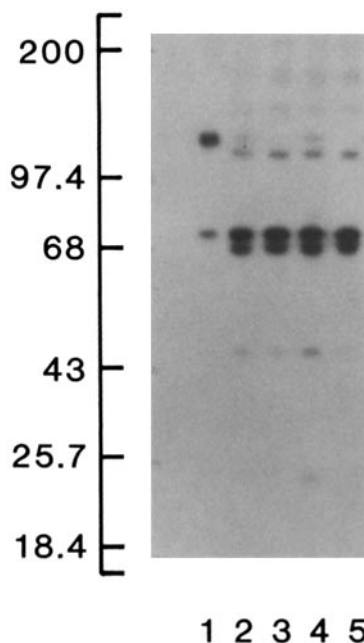


FIGURE 7. Autoradiograph of SDS-PAGE analysis of ^{125}I -C3 extracted by methylamine and SDS from *B. fragilis* and *B. thetaiotaomicron* opsonized with isolated complement proteins and EGTA-serum. Tracks 2 and 3 contain extracts from *B. fragilis* and tracks 4 and 5, extracts from *B. thetaiotaomicron*. Bacteria were opsonized with a complete mixture of the six isolated complement proteins (tracks 2 and 4) or EGTA-serum (tracks 3 and 5). Track 1 contained ^{125}I -C3. Numbers on the left refer to the positions of standard proteins having the indicated $M_r \times 10^{-3}$.

Character of Bacteria-bound C3 Fragments. The molecular form of the bacteria-bound C3 fragments generated by alternative pathway activation in isolated complement proteins and EGTA-serum was next compared. *B. fragilis* and *B. thetaiotaomicron* were opsonized for 30 min at 37°C with the complete mixture of isolated proteins or EGTA-serum and then washed. Bacteria-bound ^{125}I -C3 was extracted by treatment with methylamine and SDS, and the extracted C3 was quantitated and analyzed by SDS-PAGE and autoradiography. Of the cell-bound radioactivity, 75% was extracted from both bacterial strains opsonized with isolated proteins or serum. Autoradiographic analysis revealed that all extracts contained the β chain of C3 (70,000 mol wt), the α chain of C3b (100,000 mol wt), the 67,000-mol wt fragment of the α chain of iC3b, and high molecular weight material ($>100,000$) presumably representing the α chain of C3 bound by amide bonds to bacterial surface constituents extracted by methylamine and SDS (Fig. 7). A 45,000-mol wt band was barely visible in all extracts and presumably represents the smaller fragment of the α chain of iC3b that is poorly labeled by the iodination method used (30). Extracts from bacteria opsonized with isolated proteins also contained a faint band at 20,000 mol wt that was not observed in extracts from bacteria opsonized with EGTA-serum. A band at 110,000 mol wt was also observed that was barely visible in extracts from serum-opsonized bacteria.

TABLE III
*Semiquantitative Comparison of the Molecular Form of C3 in
 Extracts from Bacteroides Opsonized with Isolated
 Complement Proteins or EGTA-Serum*

M_r	$^{125}\text{I-C3}$ in each form			
	<i>B. fragilis</i> opsonized with		<i>B. thetaiotaomicron</i> opsonized with	
	Isolated proteins	EGTA- serum	Isolated proteins	EGTA- serum
	%			
>100,000	38	39	37	40
100,000	11	12	11	10
67,000	33	37	30	37
<60,000	18	12	22	13

The dried gel was aligned with the autoradiograph (Fig. 7), and labeled bands or areas of the gel were removed and counted in a gamma counter. The percent C3 in each form was determined by the following equation: [(cpm in band)/area]/[(cpm of bands of >100,000 M_r) + (cpm of 100,000- M_r band) + (cpm of 67,000- M_r band) + (cpm of bands <60,000 M_r)].

Semiquantitation of the 67,000 and 100,000 mol wt bands revealed that ~30–40% of the C3 extracted from both *Bacteroides* strains was in the form of iC3b and ~10% in the form of C3b (Table III). Extracts from bacteria opsonized with isolated proteins contained slightly less iC3b (67,000 mol wt band) and more low molecular weight C3 (<60,000) than extracts from serum-opsonized bacteria, presumably reflecting limited proteolytic cleavage of iC3b. The 20,000 mol wt band in extracts from bacteria opsonized with isolated proteins was not quantitated separately from the 45,000 mol wt band, because of the limited radioactivity in this section of the gel. However, the percentage of the total extracted radioactivity represented by the 20,000 mol wt band was estimated to be 4–5%. The percentage of high-molecular-weight C3 was similar in the various extracts. These results demonstrate that the predominant form of C3 bound by ester bonds to the *Bacteroides* strains through alternative pathway activation is iC3b and suggest that limited proteolytic cleavage of the bound iC3b occurred during opsonization of the bacteria with isolated proteins.

Contribution of Antibodies to Opsonization. We previously reported that antibodies in normal human serum contribute to opsonization of *Bacteroides* (10, 32, 33). These antibodies belong primarily to the IgM class and are directed against strain-specific antigenic determinants contained in the bacterial outer membrane complex (32, 33). The role of natural IgM antibodies as auxiliary factors in opsonization of *Bacteroides* by the alternative pathway was therefore explored. The purified IgM used in these studies was prepared from a pool of normal human serum, so that antibodies in this preparation would be representative of a broad range of donors. The IgM preparation was highly purified as assessed by SDS-PAGE, reacted in double immunodiffusion only with homologous anti-serum, and contained <0.8% IgG by ELISA. The *Bacteroides* strains were opsonized with purified IgM and the mixture of six alternative pathway proteins,

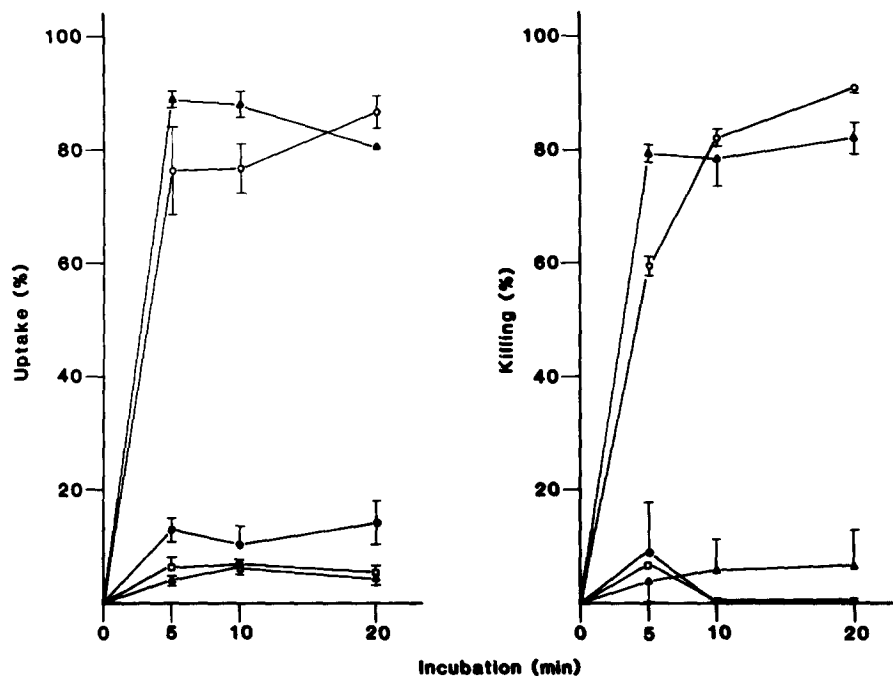


FIGURE 8. Uptake and killing by polymorphonuclear leukocytes of *B. thetaiotaomicron* opsonized with IgM and the alternative pathway. Bacteria were incubated for 30 min at 37°C with purified normal IgM and a complete mixture of the six isolated complement proteins (solid triangles), the complement mixture (solid circles) or IgM (open triangles) alone, EGTA-serum (open circles), or HBSS (open squares). After opsonization, polymorphonuclear leukocytes were added, and uptake and killing were measured at the specified time intervals. Data are expressed as mean \pm SEM of two determinations.

IgM or the complement mixture alone, or EGTA-serum. In these experiments, the bacteria were not washed after opsonization, because washing was found to induce agglutination of bacteria opsonized with purified IgM. After opsonization, polymorphonuclear leukocytes were added, and uptake and killing were measured as in preceding experiments. Opsonization of the bacteria with IgM or the alternative pathway proteins alone facilitated minimal uptake and killing by the leukocytes (Figs. 8 and 9). The combination of IgM and the complement proteins increased uptake and killing of *B. thetaiotaomicron* to the level observed with EGTA-serum (Fig. 8). In contrast, IgM and the complement proteins minimally increased uptake of *B. fragilis* and had no effect on killing of this organism. Measurement of IgM binding to the *Bacteroides* strains under the conditions used for opsonization showed that 1.08×10^4 and 9.33×10^3 IgM molecules per CFU bound to *B. thetaiotaomicron* and *B. fragilis*, respectively. Thus, the difference in opsonic activity of IgM for the two bacterial strains was not related to a quantitative difference in antibodies capable of opsonizing these strains. These results indicate that natural IgM antibodies serve as auxiliary factors in alternative pathway-mediated opsonization of *B. thetaiotaomicron*, whereas additional auxiliary factors are necessary for opsonization of *B. fragilis* by the alternative pathway.

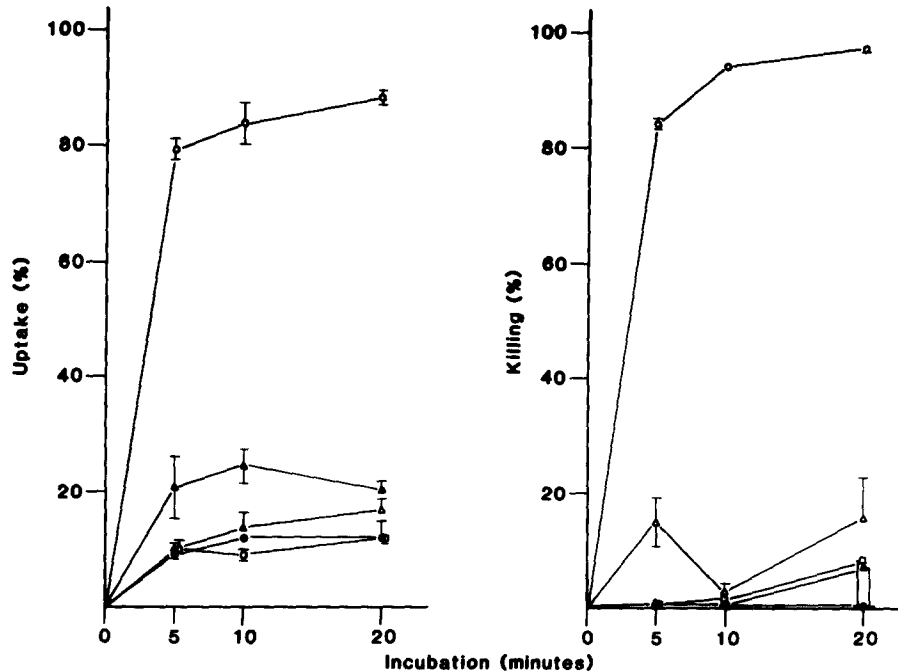


FIGURE 9. Uptake and killing by polymorphonuclear leukocytes of *B. fragilis* opsonized with IgM and the alternative pathway. Experimental conditions were as outlined in Fig. 8. Data are expressed as mean \pm SEM of two determinations.

Discussion

We have used a model system comprising the six isolated proteins of the alternative complement pathway to study the opsonic capacity of this pathway for clinical isolates of *B. fragilis* and *B. thetaiotaomicron* under physiological conditions. By using isolated alternative pathway proteins, we were able to exclude participation of other serum proteins that might influence opsonization and complicate the interpretation of our results. Rigorous criteria were used to ensure purity of the isolated complement proteins including SDS-PAGE, double immunodiffusion using a battery of monospecific antisera directed against various complement proteins, immunoglobulins, and other serum proteins, and an ELISA to rule out trace contamination with IgG. Measurement of C3 deposition using rabbit erythrocytes as the alternative pathway activator documented that the isolated proteins functioned similarly to the alternative pathway in EGTA-serum. Additional studies ruled out spontaneous activation of C3 in the model system, which can be a major problem with this system due to artifactual activation of properdin during purification.

We investigated both the early phase of opsonization involving activation of the alternative pathway by the *Bacteroides* strains resulting in C3 deposition on the bacterial surfaces and the effector phase of opsonization involving recognition of bacteria-bound C3 by polymorphonuclear leukocytes and the induction of phagocytosis and intracellular killing. Both *Bacteroides* strains were found to be potent activators of the alternative pathway as evidenced by the deposition of

$\sim 10^5$ C3 molecules per CFU when the bacteria were opsonized by isolated alternative pathway proteins. However, C3 deposition was approximately twofold greater when opsonization was mediated by EGTA-serum under similar conditions. It should be noted that the conditions used for bacterial opsonization were different from those used for measurement of C3 deposition on rabbit erythrocytes; the bacteria were added to the assays at a 10-fold lower concentration than the rabbit erythrocytes, because these conditions were found to maximize opsonization of the bacteria. In data not presented in this paper, measurement of C3 deposition using rabbit erythrocytes at the same concentration as the bacteria revealed differences in functional activity between the isolated alternative pathway proteins and EGTA-serum similar to those observed with the bacteria. These results further support the functional integrity of the model system of alternative pathway activation and suggest that factors are present in serum that augment alternative pathway activation by the *Bacteroides* strains used in our study and rabbit erythrocytes under certain experimental conditions.

Opsonization of the *Bacteroides* strains with isolated alternative pathway proteins under the same conditions used for measurement of C3 deposition did not promote adherence, uptake, or killing of the bacteria by polymorphonuclear leukocytes, whereas opsonization with EGTA-serum facilitated these events. We considered that a critical number of bacteria-bound C3 molecules exceeding 10^5 per CFU might be required for interaction of the bacteria with complement receptors on the polymorphonuclear leukocytes, and this might explain why opsonization with EGTA-serum but not isolated proteins was effective in facilitating adherence. To test this hypothesis, we opsonized the bacteria twice with isolated complement proteins and achieved numbers of bound C3 molecules comparable to those obtained upon opsonization of the bacteria with EGTA-serum. However, the increase in bound C3 had a minimal effect on adherence of the bacteria to polymorphonuclear leukocytes. These findings indicate that the concentration of bacteria-bound C3 is not the sole determinant in opsonic recognition of *Bacteroides* by polymorphonuclear leukocytes.

To determine whether protease inhibitors or other factors in serum protected bacteria-bound C3 from the action of factor I or bacterial proteases, the molecular form of C3 bound to bacteria opsonized with isolated alternative pathway proteins and EGTA-serum was compared. Radioiodinated bound C3 was extracted from the opsonized bacteria using methylamine and SDS. The combination of nucleophilic agent and detergent has been previously shown to be highly effective in releasing covalently bound and hydrophobically associated C3 from bacteria (30), and this was confirmed by our studies. Comparable amounts of C3 were extracted by this treatment from bacteria opsonized with isolated proteins and EGTA-serum. The C3 extracts were analyzed by SDS-PAGE and autoradiography, which enabled separation and semiquantitation of the various C3 fragments in the extracts. The predominant form of C3 in extracts from both *Bacteroides* strains was iC3b. C3b was also present in all extracts but in lesser amount. A low molecular weight cleavage fragment of C3 migrating at $\sim 20,000$ was detected in extracts from bacteria opsonized with isolated proteins, and the percentage of iC3b in these extracts was slightly lower than in extracts from serum opsonized bacteria. These observations suggest that limited proteolytic

cleavage of iC3b occurred in the model system of isolated proteins because of the absence of protease inhibitors or other factors present in serum. The cleavage fragment formed is smaller than the tryptic cleavage fragments, C3dg and C3d. It is doubtful that this mechanism can entirely account for the opsonic incapacity of the isolated proteins, because proteolysis was so limited.

In a recent report, Newman et al. (34) have shown that C3b is the predominant form of C3 bound to various bacterial pathogens after opsonization with serum. The absence of iC3b was striking and suggestive that proteolysis of C3b was not a typical finding under short-term incubation. Our experiments, also using a brief incubation period for opsonization, suggest that the *Bacteroides* isolates used in our study were decidedly different from the bacteria used in Newman's study, because they were predominantly coated with iC3b. This interesting difference in results may be related to protease activity associated with *Bacteroides*, characteristics of the cell envelope of these bacteria, or to other undefined factors.

It is well recognized that C3b and iC3b when bound to sheep erythrocytes adhere to specific receptors for these ligands on polymorphonuclear leukocytes, without participation of other factors (35, 36). However, there is minimal information available regarding the relative opsonic activities of C3b and iC3b when bound to bacteria. We previously demonstrated that the presence of these C3 cleavage fragments on a laboratory isolate of *Escherichia coli* facilitated adherence of the bacteria to polymorphonuclear leukocytes and also triggered uptake and intracellular killing (37). iC3b was found to be only slightly less effective than C3b in facilitating adherence of the *E. coli* strain to the leukocytes. In the present study, the reason why *Bacteroides* bearing a mixed population of iC3b and C3b did not adhere to polymorphonuclear leukocytes is speculative at present. One possible explanation is that the C3 fragments may have been impeded from interacting with the polymorphonuclear leukocytes possibly because of the physical characteristics of the moieties to which the fragments bind or to their topographical location in the bacterial cell envelope. A second explanation is that iC3b when bound to certain structures may require an auxiliary serum factor for avid binding to CR3. In this regard, there is evidence in the recent studies of Ross et al. (38) that CR3 contains two recognition sites, one for binding of iC3b and a second with lectinlike properties that binds zymosan. This latter site may also be involved in recognition of structurally similar bacterial constituents, and the *Bacteroides* strains used in our study may not possess these constituents.

Our study investigated the role of natural IgM antibodies as auxiliary factors in opsonization of *Bacteroides* by the alternative pathway. These antibodies were found to act synergistically with the alternative pathway to opsonize *B. thetaiotaomicron* as effectively as EGTA-serum. However, similar numbers of IgM antibodies directed against *B. fragilis* failed to effect opsonization of this organism by the alternative pathway. Additional factors present in normal serum are necessary for alternative pathway-mediated opsonization of *B. fragilis*, a finding that may be of significance in understanding the greater virulence of this species as compared with *B. thetaiotaomicron*. Opsonization of *B. fragilis* may be dependent on IgG antibodies, although antibodies of this class reactive with *Bacteroides* are not usually present in normal serum (39). Other serum factors that may

contribute to opsonization of *B. fragilis* are the membrane attack complex of complement, fibronectin, and C-reactive protein. The role of these factors as auxiliary opsonins for *Bacteroides* is currently under investigation in our laboratory.

The antibody independence of alternative pathway activation by rabbit erythrocytes (26, 28, 40), zymosan (41), *E. coli* (31, 37), Raji cells (42), measles virus-infected cells (43), and immune complexes (44) has been documented in other studies using similar model systems of isolated alternative pathway proteins. Certain cytotoxic effector functions of the alternative pathway also appear to occur independently of antibody (26, 31, 42). For others, such as lysis of measles virus-infected cells and complement-mediated killing of *Hemophilus influenzae*, IgG antibodies are necessary for induction of the lytic phase (43, 45). Our results have demonstrated that *B. fragilis* and *B. thetaiotaomicron*, like other potent activators of the alternative pathway, activate this system without a requirement for antibody. However, this activation process and the associated deposition of C3 cleavage fragments on the bacterial surface is not sufficient by itself to facilitate adherence, uptake, and killing of the bacteria by polymorphonuclear leukocytes. The effector phase of opsonization, like cytolysis, is an alternative pathway-dependent function that can require auxiliary serum factors. The concept that the alternative pathway usually does not require auxiliary serum factors applies only to the activation phase and should not be construed as a general concept applying to effector functions as well.

Summary

Opsonization of clinical isolates of *B. fragilis* and *B. thetaiotaomicron* with the six isolated proteins of the alternative complement pathway under physiological conditions resulted in considerable C3 deposition on the bacterial surfaces. The time course of C3 deposition was similar to that observed in EGTA-serum; however, the magnitude of C3 deposition was twofold greater in EGTA-serum. Opsonization of the bacteria with the isolated alternative pathway proteins failed to promote adherence, uptake, or killing by polymorphonuclear leukocytes, whereas opsonization of the bacteria with EGTA-serum facilitated these events. The difference in opsonic capacity of isolated proteins and EGTA-serum was not related to the quantitative difference in C3 deposition, because repeated opsonization of the bacteria with isolated proteins resulting in C3 deposition comparable to that observed in EGTA-serum only minimally increased adherence of the bacteria to polymorphonuclear leukocytes. SDS-PAGE and autoradiographic analysis of C3 extracted from bacteria opsonized with isolated proteins or EGTA-serum using methylamine and SDS demonstrated that the predominant form of C3 bound by ester bonds under both sets of conditions was iC3b. A low molecular weight C3 cleavage fragment was detected in extracts from bacteria opsonized with isolated proteins, but it accounted for only a minor fraction of the bound C3. The results of our study demonstrate that the early phase of opsonization involving activation of the alternative pathway by *B. fragilis* and *B. thetaiotaomicron* and resultant C3 deposition on the bacterial surfaces does not require auxiliary serum factors, but the effector phase of opsonization of these bacteria involving recognition of bacteria-bound C3 by polymorphonuclear

leukocytes and the induction of phagocytosis and intracellular killing is dependent on such factors. Natural IgM antibodies serve as auxiliary factors in opsonization of *B. thetaiotaomicron* by the alternative pathway, whereas additional serum factors are required for alternative pathway-mediated opsonization of *B. fragilis*.

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