INFECTION AND IMMUNITY, Oct. 1985, p. 123–129
0019-9567/85/100123-07$02.00/0

Vol. 50, No. 1

Sensitivity of *Capnocytophaga* Species to Bactericidal Properties of Human Serum

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Received 15 May 1985/Accepted 28 June 1985

***Capnocytophaga* is a newly described genus of gram-negative bacteria which can cause serious oral and extraoral infections in the susceptible host. In the present study, sensitivity of *Capnocytophaga* spp. to the bactericidal properties of human serum was investigated. Laboratory strains representative of *Capnocytophaga sputigena*, *C. ochracea*, and *C. gingivalis* and seven oral isolates of *Capnocytophaga* spp. obtained in primary culture were determined to be sensitive to killing by pooled normal serum. In contrast, little or no killing of *Capnocytophaga* spp. was observed when these organisms were incubated in the presence of hypogammaglobulinemic serum despite evidence for alternative pathway activation. However, hypogammaglobulinemic serum could be reconstituted to bactericidal activity by the addition of the immunoglobulin M fraction of normal serum. *Capnocytophaga* spp. failed to activate the classical pathway in hypogammaglobulinemic serum, thus ruling out an antibody-independent mechanism of killing. In contrast, good correlation was observed between serum-mediated killing and antibody-dependent classical pathway consumption. These results indicate that complement in the presence of bactericidal antibody may be an important determinant of host resistance to intra- and extraoral infections caused by *Capnocytophaga* spp.**

There is considerable evidence indicative of a significant role of gram-negative bacteria in the etiology of various clinical forms of human periodontal disease (23). In some instances, associations between specific gram-negative bacterial infection and discrete forms of gingivitis and periodontitis have been made (23, 30). In addition, certain oral bacteria, including *Actinobacillus actinomycetemcomitans*, have been implicated in serious extraoral infections (30).

Capnocytophaga is a newly recognized genus of capnophilic gram-negative bacteria which comprises part of the normal flora of dental plaque (12) but which appears to be involved in the pathogenesis of periodontal disease in immunocompromised hosts. These organisms are among the predominant cultivable flora in periodontal lesions of patients with juvenile-onset diabetes (11) and in patients whose neutrophils are characterized by adherence or chemotactic defects or both (2, 22). *Capnocytophaga* bacteremia has been reported in several patients with granulocytopenia (4, 5). The results of a recent retrospective study indicate, however, that *Capnocytophaga* spp. may cause bacteremic disease in both nonimmunocompromised and immunocompromised patients (17). The source of *Capnocytophaga* infections in both groups appeared to be the oral cavity.

Despite recognition of the role of *Capnocytophaga* spp. in both oral and extraoral infections, knowledge of the interaction between these organisms and host defense systems is limited. In particular, the nature and consequence of the interaction between *Capnocytophaga* spp. and the antibody-complement-phagocyte axis are undefined. Recent evidence indicates that complement may be important, at least in part, in promoting the migration of leukocytes into the junctional epithelium of dogs after topical application of sonicates of *Capnocytophaga ochracea* (28).

Numerous species of gram-negative bacteria have been shown to exhibit sensitivity to the direct bactericidal or bacteriolytic properties of serum (27). Such serum bactericidal activity is associated with activation of either the classical or the alternative complement pathways or both. Resistance of bacteria to complement-mediated killing has been suggested to be a significant factor in the ability of an organism to persist in blood (19). In view of the capacity of oral *Capnocytophaga* spp. to cause bacteremia in immunocompromised and, to a lesser extent, nonimmunocompromised patients, it was of interest to define the serum sensitivity of these organisms. The purpose of the present study was to characterize the in vitro susceptibility of laboratory strains and oral isolates of *Capnocytophaga* spp. to the bactericidal properties of human serum. Our results indicate that *Capnocytophaga* spp. are markedly sensitive to serum-mediated killing. Further, a requirement for bactericidal antibody in promoting such killing is indicated.

MATERIALS AND METHODS

Sera. Serum was obtained from healthy adult volunteers and pooled, and samples were stored at −70°C. Serum from two patients with common variable hypogammaglobulinemia was kindly provided by Madeline Lillie, Erie County Medical Center, Buffalo, N.Y. Quantitative immunoglobulin levels for these two patients were as follows (milligrams per deciliter): patient 1, immunoglobulin G (IgG), 239; IgA, 7; IgM, 23; patient 2, IgG, 187; IgA, 13; IgM, 25 (normal levels, IgG, 1,158 ± 305; IgA, 99 ± 27; IgM, 200 ± 61). Levels of complement proteins C3, C4, and factor B in serum were determined by radial immunodiffusion (complement multitest kit; ICL Scientific, Fountain Valley, Calif.) and were within normal limits. Functional capacity of the classical and alternative complement pathways of the hypogammaglobulinemic sera was confirmed by hemolytic assays with sensitized (with rabbit anti-sheep hemolysin; Difco Laboratories, Detroit, Mich.) sheep erythrocytes (classical pathway) or with unsensitized rabbit erythrocytes (alternative pathway) as described previously (29). Immunoelectrophoresis of native hypogammaglobulinemic serum yielded a single precipitin line against goat anti-human factor B (Atlantic Antibodies, Scarborough, Maine), whereas inulin-activated or zymosan-activated hypogammaglobulinemic serum exhib-

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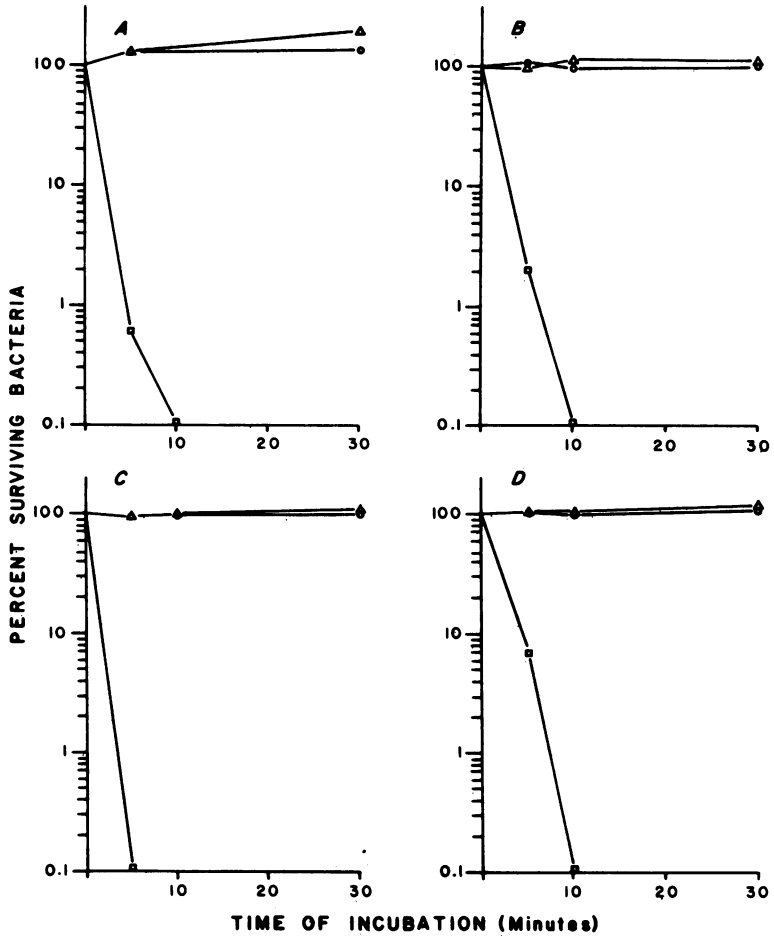

FIG. 1. Sensitivity of *Capnocytophaga* spp. to the bactericidal properties of pooled human serum. *C. sputigena* (B), *C. gingivalis* (C), and *C. ochracea* (D) were incubated with buffer alone (○), 10% native serum (□), or 10% heat-inactivated serum (△). The kinetics of killing of a known serum-sensitive organism, *S. minnesota* R595 (A), are provided for comparative purposes. Results are the means of triplicate samples removed from the incubation mixtures at specified intervals.

ited two additional cleavage products with a line of identity with native factor B.

The IgG- and IgM-containing fractions of pooled normal human serum were prepared by conventional methods. Briefly, the IgG fraction was obtained by DEAE-cellulose chromatography of an ammonium sulfate precipitate of human serum. Human myeloma IgG (subclass 1) was prepared in an identical manner. IgM was prepared by passage of intact serum over a Sephadex G-200 column. The void volume was collected, fractions containing IgM were identified by Ouchterlony analysis, and purity was assessed by immunoelectrophoresis with anti-whole human serum. IgM from a patient with Waldenström's macroglobulinemia was prepared in a similar manner, except that the serum was subjected to ammonium sulfate precipitation before application to the Sephadex G-200 column.

Serum antibodies to formalinized *Capnocytophaga* spp. were assessed by enzyme-linked immunosorbent assay as described by Mouton and co-workers (14).

Bacteria. *C. sputigena* (ATCC 33123), *C. gingivalis* (ATCC 33124), and *C. ochracea* (ATCC 27872, formerly *Bacteroides ochraceus*) were obtained from the American Type Culture Collection, Rockville, Md. Fresh clinical isolates of *Capnocytophaga* spp. were obtained by subgingival plaque sampling procedures as previously described (11). Criteria used in identification of *Capnocytophaga* spp. included the following: benzidine-positive reactivity, indole-negative reactivity, positive surface translocation, and failure to produce butyric acid as a major end product of fermentation. The serum-sensitive bacterium *Salmonella minnesota* R595 was the generous gift of David C. Morrison, Emory University, Atlanta, Ga. The serum-resistant organism *A. actinomycetemcomitans* Y4, originally obtained from S. Socransky of the Forsyth Dental Center, is maintained in our culture collection. The latter two organisms were used in initial studies designed to validate the bactericidal assay conditions, as described below. Bacteria were maintained on 5% sheep blood agar containing 5 μg of equine hemin III (Sigma Chemical Co., St. Louis, Mo.) per ml, 0.001% menadione (Sigma), and 0.1% yeast extract (Difco) and incubated at 37°C in 5% CO_2. On the day before each assay, overnight cultures of bacteria were prepared in either 3% tryptic soy broth (*Capnocytophaga* spp. and *S. minnesota*) or NIH thioglycollate broth (*A. actinomycetemcomitans*). The next day, 0.5-ml samples were transferred to fresh media and incubated at 37°C in a 5% CO_2 atmosphere for several additional hours to achieve an appropriate bacterial density (optical density at 650 nm equal to 0.1, corresponding to approximately 10^8 organisms per ml). The bacteria were recovered in logarithmic phase and diluted

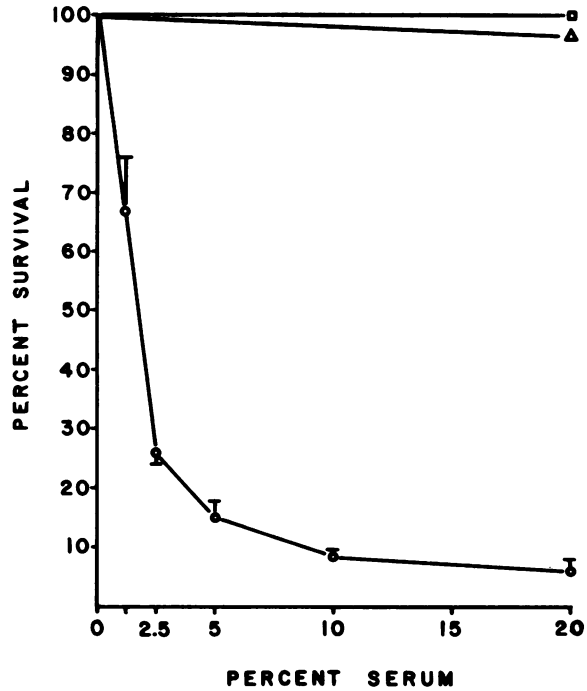

FIG. 2. Effect of serum concentration on the sensitivity of *C. sputigena* to serum bactericidal activity. Bacteria were incubated for 30 min at 37°C in the presence of specified serum concentrations. Each point represents the mean (± standard deviation) of triplicate samples. Symbols: □, GVB; △, heated normal human serum; ○, normal human serum.

1:100 into barbital buffer (ionic strength, 0.15 [pH 7.4]) containing 0.1% gelatin, 1.0 mM $MgCl_2$, and 0.15 mM $CaCl_2$ (GVB) without prior centrifugation.

Serum bactericidal assay. All assays were performed in sterile 12-by-75-mm polystyrene tubes (Falcon; Becton, Dickinson Labware, Oxnard, Calif.) which were incubated in a 37°C shaking water bath. Serum samples were thawed immediately before use and either used directly or heat inactivated at 56°C for 30 min. Samples (0.1 ml) of bacterial suspension were transferred to culture tubes containing GVB and the indicated concentration of native or heat-inactivated serum in a final volume of 1.0 ml. At specified intervals, the samples were removed, serially diluted, and plated (0.05 ml) onto sheep blood agar. After a 2- to 3-day period of incubation at 37°C in 5% CO_2, CFU were counted, and the corresponding reduction in bacterial viability was calculated. In some instances, serum was absorbed two times at 0°C for 30 min with 10^9 bacteria per ml and subsequently filter sterilized with a membrane filter (pore size, 0.22 μm; Millipore Corp., Bedford, Mass.).

Complement assays. Consumption of serum complement by indicated bacteria was monitored by using inhibition hemolytic assays as described elsewhere (29). Briefly, 0.15 ml of GVB containing 7.5×10^8 bacteria were incubated with an equal volume of a 1/8 dilution of serum for 30 min at 37°C. The sera were centrifuged, and 0.2-ml samples were transferred to culture tubes containing 5×10^8 antibody-coated sheep erythrocytes in a volume of 0.25 ml of GVB. After an additional 30-min incubation at 37°C in a shaking water bath, the reactions were terminated by adding 2 ml of ice cold 0.9% NaCl. The tubes were centrifuged at 4°C for 7 min at 850 × *g*. Absorbancy of the supernatant fractions was measured at 412 nm, and complement consumption was determined by inhibition of hemolysis as calculated by using the absorbancy of supernatants from tubes containing GVB-treated serum. Complement activation is expressed as the percent consumption of available hemolytic activity.

Immunoelectrophoresis. Conversion of factor B in serum after incubation with bacteria was determined as follows: 0.05 ml of bacterial suspension in 0.9% NaCl was incubated with an equal volume of serum for 30 min at 37°C. After centrifugation, samples were removed, electrophoresed in 1.2% agarose (SeaKem; FMC Corp., Marine Colloids Div., Rockland, Maine) for 2 h at 6.7 V/cm, and developed with goat anti-human factor B (Atlantic Antibodies).

RESULTS

Sensitivity of *Capnocytophaga* spp. to serum bactericidal action. The sensitivity of logarithmic-phase cultures of *C. sputigena*, *C. gingivalis*, and *C. ochracea* to the bactericidal properties of pooled human serum was examined. All three *Capnocytophaga* species were markedly sensitive to serum-mediated killing (Fig. 1). Viability of *Capnocytophaga* spp. declined by more than 90% after a 30-min incubation in 10% human serum. No loss of viability was evident after exposure of bacteria to heat-inactivated serum, indicating that heat-labile serum components were required for killing. The kinetics of killing of *Capnocytophaga* spp. by serum closely paralleled those of a known serum-sensitive deep rough mutant bacterium *S. minnesota* R595 (Fig. 1A). Under identical conditions, however, no killing of another periodontal organism, *A. actinomycetemcomitans*, was observed after incubation with 20% serum for periods up to 1 h (data not shown). The latter finding is in agreement with the earlier observations of Sundqvist and Johansson (25).

Preliminary studies indicated that a serum concentration of 10% (vol/vol) was optimal for serum killing of *Capnocytophaga* spp. The extent of killing of *C. sputigena* increased as the serum concentration was raised from 1.25 to 10% serum (Fig. 2). A similar pattern was observed for serum killing of *C. ochracea* and *C. gingivalis*.

Serum sensitivity of fresh *Capnocytophaga* isolates was also determined. Oral isolates obtained in primary culture had a pattern of serum sensitivity similar to that of the ATCC

TABLE 1. Serum sensitivity of fresh oral isolates of *Capnocytophaga* spp.[a]

Strain	Patient source	Percent survival after 30 min
CPCL1	Localized juvenile periodontitis	<1.0
CPCL2	Localized juvenile periodontitis	6.6
CPCW1	Localized juvenile periodontitis	19.2
CPLS1	Adult periodontitis	<1.0
CPLS2	Adult periodontitis	<1.0
CPLJ1	Normal	<1.0
CPLJ2	Normal	<1.0

[a] Bacteria were incubated for 30 min at 37°C in the presence of 10% pooled human serum, after which samples were removed, serially diluted, and plated onto blood agar plates. After incubation for 2 to 3 days in a humidified CO_2 incubator, CFU per plate were determined (each count performed in duplicate). Percent survival was determined as a function of viability at $t = 0$ min (adjusted to an initial density of 2×10^5 to 3×10^5 CFU/ml). Under standard assay conditions, the limit of sensitivity of CFU detection (one colony per plate) corresponds to 2×10^3 CFU/ml or 1.0% of the initial inoculum. Plates showing no growth after 3 days of incubation are reported as <1.0% survival. No loss of viability was observed in tubes containing buffer alone or in tubes supplemented with 10% heat-inactivated serum.

strains examined (Table 1). One isolate (strain CPCW1) was, however, killed at a reduced rate compared with the other laboratory and clinical strains.

Requirement for antibody in serum-mediated killing of *Capnocytophaga* spp. Complement-mediated killing of numerous species of gram-negative bacteria is dependent upon the presence of bactericidal antibody. To assess the role of antibody in facilitating serum killing of *Capnocytophaga* spp., bactericidal assays were done with hypogammaglobulinemic serum as a complement source. Little or no killing of *Capnocytophaga* spp. resulted after a 30-min exposure of these organisms to 10% hypogammaglobulinemic serum, in contrast to the marked killing observed in 10% normal human serum (Table 2). Significant killing of *S. minnesota* R595 by either 10% normal or hypogammaglobulinemic serum occurred during this same period. Repeated absorption of hypogammaglobulinemic serum at 0°C with the autologous organism did not reduce killing, consistent with earlier findings that killing of *S. minnesota* R595 proceeds via the classical pathway but is antibody independent (3). With 10% hypogammaglobulinemic serum as a source of complement, the relative efficacy of the IgG and IgM fractions of normal human serum in reconstituting bactericidal activity against *C. sputigena* was examined. IgM was considerably more effective than IgG in reconstituting bactericidal activity of hypogammaglobulinemic serum (Fig. 3). In contrast, the IgM fraction obtained from a patient with Waldenström's macroglobulinemia failed to promote killing of *C. sputigena* at concentrations up to 100 μg/ml (data not shown). Similarly, myeloma IgG lacked detectable bactericidal activity at concentrations up to 1 mg/ml. None of the IgG or IgM fractions directly influenced bacterial viability. These results indicate that serum-mediated killing of *Capnocytophaga* spp. requires the presence of antibody and does not proceed to any appreciable degree via an antibody-independent classical pathway route.

It was of interest to determine whether the observed differences in killing of *Capnocytophaga* spp. by normal and hypogammaglobulinemic sera corresponded to differences in the levels of specific antibody in these two serum sources.

TABLE 2. Serum bactericidal activity of normal and hypogammaglobulinemic serum against *Capnocytophaga* species

Species	Assay conditions[a]	CFU/ml at:	
		0 min	30 min
C. sputigena	Buffer alone	2.9×10^5	2.2×10^5
	10% NHS	2.7×10^5	$<2.0 \times 10^3$[b]
	10% HγS	2.5×10^5	2.4×10^5
	10% heated NHS	2.6×10^5	2.7×10^5
C. gingivalis	Buffer alone	7.5×10^5	7.3×10^5
	10% NHS	7.5×10^5	2.0×10^3
	10% HγS	6.5×10^5	6.4×10^5
	10% heated NHS	6.6×10^5	6.9×10^5
C. ochracea	Buffer alone	3.6×10^5	3.7×10^5
	10% NHS	3.6×10^5	$<2.0 \times 10^3$
	10% HγS	3.8×10^5	2.6×10^5
	10% heated NHS	4.1×10^5	3.9×10^5
S. minnesota R595	Buffer alone	4.6×10^5	6.4×10^5
	10% NHS	4.9×10^5	$<2.0 \times 10^3$
	10% HγS	6.0×10^5	$<2.0 \times 10^3$
	10% absorbed HγS	5.7×10^5	$<2.0 \times 10^3$
	10% heated NHS	6.1×10^5	9.4×10^5

[a] NHS, Normal human serum; HγS, hypogammaglobulinemic serum.

[b] Under standard assay conditions, a single CFU per plate corresponds to 2×10^3 CFU/ml. Accordingly, plates showing no colony formation are designated as $<2 \times 10^3$ CFU/ml.

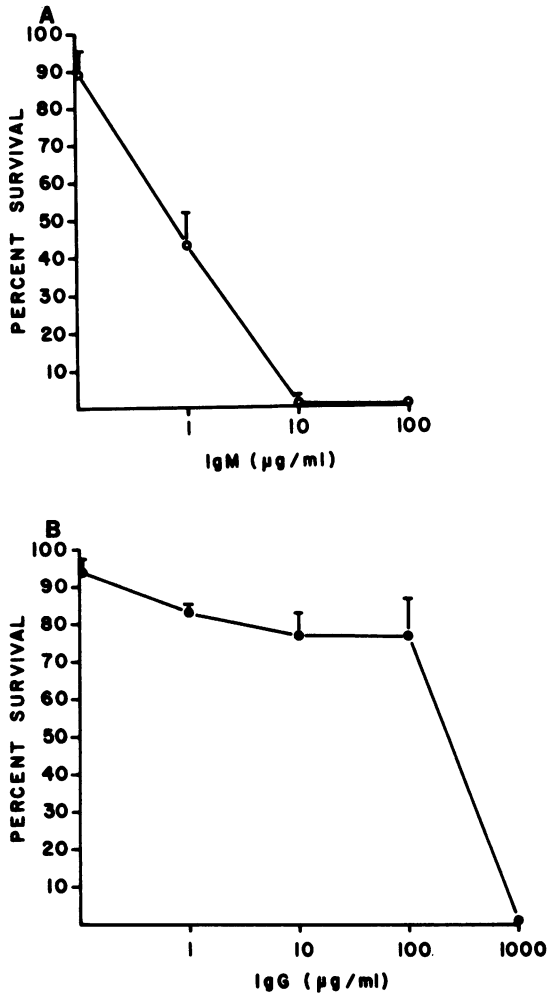

FIG. 3. Role of immunoglobulin in complement-mediated killing of *C. sputigena*. Bacteria were incubated at 37°C in 10% hypogammaglobulinemic serum (as a complement source), to which was added various concentrations of IgM (A) or IgG (B) from normal human serum. After a 30-min incubation, bacterial viability was determined. Values represent the means (± standard deviation) of triplicate samples.

Accordingly, specific IgG and IgM antibodies directed toward these organisms were measured with an enzyme-linked immunosorbent assay. Normal serum contained detectable amounts of specific IgG and IgM antibodies against all three *Capnocytophaga* species (Table 3). In contrast, hypogammaglobulinemic serum contained significantly reduced levels of specific antibody against these organisms.

Serum complement activation by *Capnocytophaga* spp. The extent to which bactericidal antibody contributes to serum killing of *Capnocytophaga* spp. by virtue of an ability to enhance complement activation was examined. Bacteria were incubated for 30 min in the presence of normal or hypogammaglobulinemic serum. Residual complement activity was then determined by a standard hemolytic assay which principally reflects consumption of the early classical pathway components. All three *Capnocytophaga* spp. consumed complement via the classical pathway in normal serum (Table 4). In contrast, virtually no complement activation by *Capnocytophaga* spp. was observed in hypogammaglobulinemic serum. Nevertheless, *S. minnesota* R595

TABLE 3. Antibodies to *Capnocytophaga* spp. in normal and hypogammaglobulinemic serum

Antibody source	Isotype	Serum antibody to:		
		C. ochracea	*C. gingivalis*	*C. sputigena*
Pooled normal human serum	IgG	4.8[a]	179.7	54.2
	IgM	38.9	182.0	7.7
Hypogammaglobulinemic serum[b]	IgG	3.7	0.2	<0.25
	IgM	<0.25	<0.25	<0.25

[a] Results are expressed as enzyme-linked immunosorbent assay units of IgG or IgM antibodies in the indicated test serum.
[b] Obtained from patient 1.

consumed serum complement in normal, hypogammaglobulinemic, and absorbed hypogammaglobulinemic serum, providing additional evidence that this organism fixes complement via the classical pathway and in the absence of antibody. These results are consistent with the hypothesis that bactericidal antibody promotes serum killing of *Capnocytophaga* spp. by amplifying complement activation via the classical pathway.

Activation of the alternative pathway contributes to the killing of certain species of gram-negative bacteria. We wished to determine whether *Capnocytophaga* spp. resist killing in antibody-deficient serum because of an inability to activate complement via the alternative pathway. However, direct evidence of such a capacity is provided by the demonstration that *Capnocytophaga* spp. activate factor B of the alternative pathway (Fig. 4). Conversion of factor B to two cleavage fragments was demonstrated in both normal (Fig. 4) and hypogammaglobulinemic serum (data not shown). Thus, although *Capnocytophaga* spp. activated the alternative pathway, such activation appeared to be either ineffective or minimally effective in reducing bacterial viability during short-term (30-min) exposure to serum.

DISCUSSION

Capnocytophaga spp. are facultatively anaerobic, capnophilic gram-negative bacilli which constitute part of the oral flora in both normal individuals and in patients with localized juvenile periodontitis (12). Recent studies indicate that these organisms are capable of causing serious nonoral infections in both immunocompromised (4, 5, 17) and nonimmunocompromised (17) hosts. Notably, bacteremia occurred in both the immunocompromised and nonimmunocompromised patient groups. In all patients with *Capnocytophaga* infection, an oral source of the organism was suggested.

Information regarding host defense mechanisms which may be operative in restricting dissemination of oral *Capnocytophaga* spp. to nonoral sites is unavailable. Bacteremia caused by *Capnocytophaga* spp. was more common in immunocompromised granulocytopenic patients (17), suggesting that neutrophilic granulocytes may play a significant role in host defense against these organisms. However, the observation that bacteremias occurred, albeit to a lesser extent, in nonimmunocompromised patients indicated that other immune defense mechanisms may be equally important in effecting removal of *Capnocytophaga* spp.

Whereas many species of gram-negative bacteria are sensitive to the lethal properties of serum, organisms isolated from blood are often serum resistant (19, 27). Recovery of *Capnocytophaga* spp. from blood cultures of immunocompromised and nonimmunocompromised patients raised the possibility that these organisms are similarly resistant to serum bactericidal action. The purpose of the present investigation was, therefore, to define the sensitivity of *Capnocytophaga* spp. to the bactericidal action of human serum.

The results of the present study indicated that laboratory strains and fresh oral isolates of *Capnocytophaga* spp. are markedly sensitive to the bactericidal properties of human serum. Hypogammaglobulinemic serum failed to support killing of these organisms, thereby indicating a requirement for bactericidal antibody. Although the serum concentrations of bactericidal antibody in normal and hypogammaglobulinemic serum were not determined, levels of specific IgG and IgM antibody against *Capnocytophaga* spp.

TABLE 4. Activation of complement in normal and hypogammaglobulinemic sera by *Capnocytophaga* spp.

Antibody source	% Reduction in hemolytic activity by[a]:			
	C. sputigena	*C. ochracea*	*C. gingivalis*	*S. minnesota* R595
Normal human serum	98.2	98.8	98.9	98.4
Hypogammaglobulinemic serum	0.1	2.1	0.1	98.6
Absorbed hypogammaglobulinemic serum[b]	ND[c]	ND	ND	98.9

[a] Complement consumption was determined by hemolytic assay, as described in the Materials and Methods.
[b] Serum was absorbed two times with 10^9 bacteria per ml at 0°C for 30 min and was subsequently filtered with a 0.22-μm-pore-size filter. This procedure reduced the hemolytic activity of the serum by less than 5%.
[c] ND, Not determined.

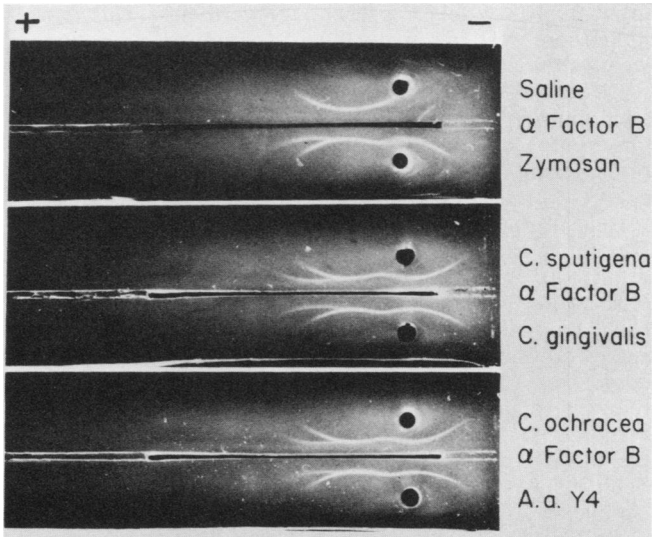

FIG. 4. Cleavage of factor B in pooled human serum by *Capnocytophaga* spp. Portions (50 μl) of 0.9% NaCl alone or containing 5×10^7 bacteria were incubated with an equal volume of pooled human serum for 30 min at 37°C. The samples were then centrifuged and electrophoresed as described in the Materials and Methods. Slides were developed with goat anti-human factor B.

were markedly reduced in hypogammaglobulinemic serum compared with normal serum. No loss of bacterial viability was observed in heat-inactivated (56°C, 30 min) normal human serum. Additional evidence to support an antibody-dependent mechanism of serum killing derives from our finding that hypogammaglobulinemic serum could be reconstituted to bactericidal activity by addition of the IgM (and, to a much lesser extent, the IgG) fraction of pooled human serum. The inability of the IgM fraction of serum from a patient with Waldenström's macroglobulinemia to reconstitute hypogammaglobulinemic serum would argue against nonspecific binding of immunoglobulin to the bacterial surface. These findings indicate that both heat-labile (complement) components and antibody are required for optimal killing of *Capnocytophaga* spp. by serum.

The *Capnocytophaga* cell envelope possesses constituents capable of activating complement via the classical and alternative pathways (15). We sought to determine which pathway(s) of complement activation mediates serum killing of *Capnocytophaga* spp. The alternative pathway, in the complete absence of antibody, is bactericidal for certain gram-negative bacteria (21), whereas in other instances immunoglobulin is required to promote alternative pathway-mediated killing (7). As an example of the latter case, IgG or $F(ab')_2$ fragments were found to mediate killing of a serum-sensitive *Escherichia coli* O111B4 strain by the alternative pathway without increasing terminal complement component deposition on the bacterial surface (7).

Although no killing of *Capnocytophaga* spp. was observed in hypogammaglobulinemic serum despite direct evidence (factor B cleavage) for alternative pathway activation, it is possible that bactericidal antibody may facilitate killing of these organisms via the alternative pathway in a manner analogous to that described for *E. coli* O111B4. However, two points would tend to disfavor this mechanism. First, the alternative pathway of complement is reported to be highly sensitive to dilution. Previous studies by Schreiber et al. (21) indicated that the bactericidal activity of a mixture of 11 isolated alternative pathway proteins became insignificant when diluted to 1:16 physiologic concentration (corresponding to approximately 6% serum). Nevertheless, *Capnocytophaga* spp. were effectively killed at serum concentrations in which alternative pathway function is likely minimal (1.25 to 5.0% serum). Second, a strong correlation between killing and consumption of classical pathway components was observed. Thus, it appears likely that bactericidal antibody promotes killing of *Capnocytophaga* spp. by increasing the extent of classical pathway activation.

It is intriguing that *Capnocytophaga* spp. activate the alternative pathway of complement without a detectable loss of viability. Although no definitive explanation for this observation can be given at present, several possibilities exist. First, alternative pathway activation may proceed at a sufficiently slow rate to permit shedding of membrane attack complexes before injury can occur. In this regard, it is relevant to point out that *Capnocytophaga* spp. produce numerous outer membrane vesicles (blebs) which are morphologically quite similar to lipopolysaccharide vesicles (6). Such vesicles may serve either to remove membrane attack complexes from the bacterial surface or, alternatively, to fix complement at a site remote from the organism itself. Second, it is possible that proteolytic enzymes released by these organisms may activate complement. However, we determined that the structure(s) on the surface of these organisms responsible for alternative pathway activation is resistant to heat inactivation (100°C, 15 min). Thus, an enzymatic basis for alternative pathway activation seems unlikely. Third, it is conceivable that these bacteria may remove terminal components from their surface by internalization rather than by shedding.

The basis for the marked serum sensitivity of *Capnocytophaga* spp. is not, at present, understood. However, it is noteworthy that these organisms exhibited a degree of serum sensitivity which resembled that of the serum-sensitive deep rough mutant *S. minnesota* R595. Rough mutants of gram-negative bacteria often exhibit greater sensitivity to serum-mediated killing than do corresponding smooth strains. It has been reported that a phenol-extracted lipopolysaccharide derived from *C. gingivalis* exhibited a pattern on sodium dodecyl sulfate-polyacrylamide gels which is typical of rough or semirough lipopolysaccharides (18). Whether serum sensitivity of *Capnocytophaga* spp. can be attributed, at least to some degree, to expression of such a lipooligosaccharide structure in the outer membrane warrants further study.

Polysaccharide-deficient lipopolysaccharides from core-defective bacterial strains can activate the classical pathway, in the absence of antibody, via direct interaction with precursor C1 (13). Certain species of gram-negative bacteria activate C1 in the absence of antibody (1, 10). Antibody-independent activation of the classical pathway can lead to effective killing of certain species of gram-negative bacteria. This mechanism appears to contribute to the serum sensitivity of core-defective rough mutant bacterial strains, including *S. minnesota* R595 (3). However, despite the apparent lipooligosaccharide character of *Capnocytophaga* endotoxin (18), antibody-independent classical pathway activation does not contribute to serum killing of *Capnocytophaga* spp., because neither bactericidal activity nor consumption of classical pathway components was evident in hypogammaglobulinemic serum.

The in vivo consequences of *Capnocytophaga*-complement interactions warrant further study. Gingival fluids contain appreciable amounts of immunoglobulin and complement proteins. Moreover, fluids obtained from the gingival crevices of patients with periodontal disease contain activation products of complement components C3, C4, and factor B (20). Although the consequences of such complement activation in fluids bathing subgingival plaque bacteria are not fully understood, it has been reported that bacteria resident in the gingival crevice are coated with antibody and with C3 (16). Complement activation in the gingival crevice and in subjacent tissues may potentially result in either (i) direct killing of susceptible organisms or (ii) opsonization of these bacteria for subsequent ingestion and killing by phagocytic cells. To date, however, few studies have examined the sensitivity of various subgingival plaque bacteria to the bactericidal properties of complement (25).

In view of the apparent protective role of antibody in host defense against *Capnocytophaga* spp., it is of interest that levels of serum antibody directed toward *Capnocytophaga* spp. are not elevated in patients with periodontal disease compared with normal individuals (26). Notably, patients with localized juvenile periodontitis, whose subgingival flora contain significant numbers of *Capnocytophaga* spp., have a decreased level and frequency of antibody response to these organisms (26). It has also been demonstrated that *Capnocytophaga* spp. produce IgA and IgG proteases (8) which may subvert local immune defenses, including antibody-dependent classical pathway activation. Other factors, including availability of complement proteins within the tissues of the periodontium and potential variations in serum

sensitivity attendant to growth phase, may also contribute to the survival of this organism.

In summary, reference strains and oral isolates of *Capnocytophaga* spp. were determined to be sensitive to the complement-dependent bactericidal activity of human serum. Killing of these organisms appeared to be mediated principally via antibody-dependent classical pathway activation. In view of the marked serum sensitivity of *Capnocytophaga* spp., it is intriguing that these organisms are capable of causing bacteremia in both immunocompromised and nonimmunocompromised patients. Whether *Capnocytophaga* strains associated with bacteremia are resistant to complement-mediated killing by serum, as might be anticipated, is currently under investigation. Nevertheless, killing of *Capnocytophaga* spp. by complement may be an important factor in host resistance against this organism, particularly within the local environment of the periodontium.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant DE06640 from the National Institute of Dental Research.

We thank M. Apicella and J. J. Zambon for critical evaluation of this manuscript, Paulette Hammond for performing the enzyme-linked immunosorbent assays, Denise Nicosia for excellent secretarial assistance, and Hector Velasco for technical illustrations.

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